The potential pathogenicity of *Dolosigranulum pigrum* in multiple sclerosis, and the occurrence of the organism in the upper respiratory tract

by

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Abstract

The bacterial toxins hypothesis in multiple sclerosis postulates that bacterial toxins from the human nasopharynx access the central nervous system and are implicated in the disease. *Dolosigranulum pigrum* was originally found in acute multiple sclerosis tissues. This study seeks to determine if the organism is found in human nasal tracts and explores the relationship between *Dolosigranulum pigrum* infection and multiple sclerosis by measuring antibody to the bacterium in multiple sclerosis patients and matched controls.

Eighty eight clinical specimens were cultured onto blood agar and analysed using rabbit anti-*Dolosigranulum pigrum* sera, and fluorescein isothiocyanate. None tested positive for *Dolosigranulum pigrum*. *Dolosigranulum pigrum* was looked for using a novel polymerase chain reaction test. Thirty samples tested positive, 17 male, 13 female, with an age range from 8 days to 74 years. Twelve were sent for sequencing, seven matched *Dolosigranulum pigrum*, two showed mixed amplicons and three produced signals not matching *Dolosigranulum pigrum*.

Sixty five multiple sclerosis sera and matched controls were tested for anti-*Dolosigranulum pigrum* using a novel enzyme linked immunosorbant assay. The multiple sclerosis group showed raised antibodies, significantly different to the controls, $p \leq 0.001$. Fifteen of the multiple sclerosis sera and controls were Western blotted. There were no common bands when compared with rabbit sera containing antibodies to *Dolosigranulum pigrum*.

This study shows that *Dolosigranulum pigrum* is found in the nasal tract. The elevated antibodies were not found to be anti-*Dolosigranulum pigrum* but their presence may be suggestive of bacterial products leaking from the nasal tract into the central nervous system. Further research into bacteria in the nasal tract is indicated. Introduction of markers into the nasal passages with subsequent tracking into the central nervous system by magnetic resonance imaging could provide evidence of a route by which bacterial molecules enter the central nervous system 'behind' the blood brain barrier.

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Abbreviations

ATCC	American type culture collection
AWO	Antral washout
BA	Blood agar
BHI	Brain heart infusion
BLAST	Basic alignment tool
bp	Base pair
CAN	*Candida* species
CHUFT	Colchester Hospital University Foundation Trust
CNS	Central nervous system
COL	Coliform
CSF	Cerebrospinal fluid
CT	Computerised tomography
d	Day
DAB	3,3′-diaminobenzadine tetrahydrochloride
DEPC	Diethylpyrocarbonate
DNA	Deoxyribose nucleic acid
dNTP	Deoxynucleotide triphosphate
EAE	Experimental allergic encephalomyelitis
EBV	Epstein Barr Virus
ECA	Ethmoid cavity swab
ECO	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
Eh	Redox potential
EIA	Enzyme immunoassay
ELISA	Enzyme linked immunosorbent assay
ETH	Ethmoid sinus fluid
FASTA	FASTALL protein system alignment software package
FITC	Fluorescein isothiocynate
h	Hours
HAS	Haemolytic streptococcus group A
HCl	Hydrochloric acid
HIN	*Haemophilus influenzae*
HLA	Human leucocyte antigen
HPC	Health Protection Council
HRP	Horse radish peroxidase
HSG	Haemolytic streptococcus group G
IgG	Immunoglobulin G
IE	Infective endocarditis
kDa	Kilo Dalton
LASER	London and South East Region
LAP	Leucine aminopeptidase
M	Mean
m	Month
min	Minutes
MHS	Major histocompatability complex

MIXCOL	Mixed coliforms
MOR	Mixed organisms
MOU	Mouth swab
MRI	Magnetic resonance imaging
MRS	Methicillin resistant *Staphylococcus aureus*
MS	Multiple sclerosis
N	Normal
NB	Newborn
NCIMB	National Collection of Industrial, Food and Marine Bacteria
NF	Normal flora
NG	No growth
NMO	Neuromyelitis optica
NMU	Nasal mucous
NPA	Nasopharyngel aspirate
NRES	National Research Ethics Service
NS	Nose swab
OD	Optical density
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD	Professional Doctorate
QAS	Quinsy aspirate
PYR	Pyrrolidonyl arylamidase
QSW	Quinsy swab
rRNA	Ribosomal ribonucleic acid
RSV	Respiratory syncitial virus
R3	Rabbit 3
R6	Rabbit 6
s	Seconds
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SDS PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SF	Skin flora
SMI	*Streptococcus milleri*
SPN	*Streptococcus pneumoniae*
TAE	Tris acetate ethylenediaminetetraacetic acid buffer
Taq	Taq polymerase
TLRs	Toll-like receptors
TEMED	Tetramethylethylenediamine
TMB	Tetramethylbenzadine buffer
TMU	Throat mucous
TS	Throat swab
TSST	Toxic shock syndrome toxin
URI	Urine
UV	Ultra violet
w	Week
y	Year

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Dissemination

Publications

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Presentations

Multiple Sclerosis part 1 – 15th March 2011, Colchester Hospital University Foundation Trust microbiology
Multiple Sclerosis part 2 – 12th May 2011, Colchester Hospital University Foundation Trust microbiology
Multiple Sclerosis part 3: The hunt for *Dolosigranulum pigrum* - 12th December 2012, Colchester Hospital University Foundation Trust microbiology
Multiple Sclerosis part 4: ELISA and Western blotting results – 25th April 2013, Colchester Hospital University Foundation Trust microbiology

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Declaration

I declare that whilst studying for the Doctorate in Biomedical Science at the University of Portsmouth I have not been registered for any other award at another university. The work undertaken for this degree has not been submitted elsewhere for any other award. The work contained within this submission is my own work and, to the best of my knowledge and belief, it contains no material previously published or written by another person, except where due acknowledgement has been made in the text.

F. M. Jorge

Dedication

Brian Martin Jorge 27/03/36 – 15/09/11

For Dad.

You will never see the finished work, never attend the party I promised you I'd hold to celebrate its completion, but without your love and support I would never have got this far.

Thinking of you now and always,

Your Loving Son,

Mark.

Foreword

"The story of multiple sclerosis is like a history of medicine in minature."
Tracy J. Putnam (1938).

Multiple sclerosis (MS) is now rarely fatal but the progressive loss of neurological function so frequently leads to overwhelming despair.

> *"Why this deliberate, slow-moving malignity? . . . I am not offering up my life willingly - it is being taken from me piece by piece, while I watch the pilfering with lamentable eyes."*
> Barbellion (1919).

The above quotation is from the diary of Bruce Cummings, under his pseudonym W.N.P Barbellion, and details his struggle with MS in the first half of the last century. The disease remains the major cause of incurable paralysis in young adults in the developed world, with an estimated 400,000 cases in Europe alone (Rotstein, Hazan, Barak & Achiron, 2006, pp. 511-516) at an estimated cost of €12.5 billion per year (Sobocki, 2007, pp. 1054-1064). MS is characterised by patches of demyelination found in the central nervous system called plaques. These plaques may be found anywhere in the brain and spinal cord but appear to have a predilection for the cervical cord, brain stem, cerebellum and optic tracts. The inflammation of acute plaques appears to be located around small veins. Clinical signs and symptoms, characterised by motor and sensory deficits are, like the lesions, separated in time and space. These symptoms may include a combination of:

- Visual – Optic neuritis, Diplopia, Nystagmus
- Motor – Muscle weakness, paralysis, spasms
- Pain – Neuropathic, Musculoskeletal
- Mobility – Ataxia, tremor, dizziness
- Cognitive – Reduced mental speed, slow and slurred speech, difficulty in learning/remembering

- Fatigue
- Bladder and bowel problems

There is no specific laboratory test for the disease and diagnosis is made clinically, aided by imaging techniques, evoked potentials and the finding of oligoclonal bands in the cerebrospinal fluid. MS can be confused with strokes, Lyme disease, chronic fatigue syndrome and other diseases. The prevalence of MS has been estimated to be 1 in 1000 and it has been calculated that 50% of patients are unable to walk without assistance 15 years after onset (Polman & Uitdehaag, 2000, pp. 490-494). Yet despite over 170 years of intensive research the cause of MS, a specific diagnostic test for it, and a treatment, has eluded medical science.

Chapter 1:
Introduction and literature review

1.1 History of multiple sclerosis

1.1.1 Naming and classifying the disease

The first depiction of the distinctive patches of demyelination seen in multiple sclerosis (MS) appeared in 1838 in an atlas of pathology published by Robert Carswell (1838), a Scottish pathologist working in Paris. Carswell, who probably never saw his subjects alive, did not include clinical details with his illustrations. A link between the disease and its unusual microscopic appearance had to wait until 1842 when Jean Cruveilhier, who was also working in Paris, published his illustrations of the lesions along with clinical histories of the cases. In the next few years further important observations were made. Eduard Reindfleisch (1863, pp. 274-483) provided further insights into the pathophysiology of MS by observing that the lesions found in MS usually developed around small cerebral blood vessels, with plaques developing from these foci of chronic recurring peri-vascular inflammation. In the same year Leyden (1863, pp. 114-116) noted that there was an excess of MS in women, with the disease first occuring in the third decade of life. He postulated that MS was precipitated by disease, exposure to damp and cold or by emotional upsets. MS was finally characterized as a disease by Jean-Martin Charcot who initially named it ‘sclerose en plaques disseminées’ in his seminal work of 1868 (pp. 557-558, 566). Charcot linked nystagmus, scanning speech and intention tremor to differentiate MS from other causes of paralysis, and then proceeded to describe the three cardinal pathological changes seen in MS: glial cell proliferation,

myelinated fibre degeneration and blood vessel changes (Figure 1.1). He speculated that some insult led to glial cell proliferation which then resulted in the demylination of nerve cells.

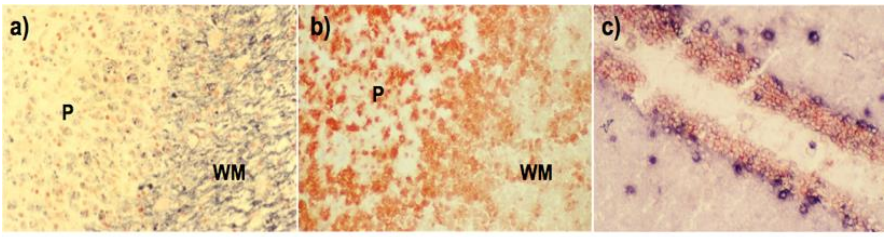

Figure 1.1. Charcot's Pathological Triad. (Courtesy F. Gay)
a) Demyelination - multiple sclerosis plaque (P) showing demyelination in white matter (WM). Luxol fast blue stain for myelin.
b) Glial cell proliferation - Macrophages in acute multiple sclerosis plaque (P). Monoclonal antibody Y1.83a, specific for macrophages + Horse Radish Peroxidase.
c) Blood vessel inflammation -Vessel traversing acute multiple sclerosis tissue. CD4+ T cells (brown) and CD8+ T cells (purple).

1.1.2 Clinical features and pathology

There are many forms of MS (Whitaker & Mitchell, 1997, pp. 3-19) but the relapsing-remitting type, where attacks are followed by periods of remission lasting from a few days to months, is the commonest presentation (Figure 1.2A). These remissions may occasionally result in a complete recovery each time but normally the outcome is an improvement rather than the disappearance of symptoms. A sub-group of patients with relapsing-remitting disease show a relatively benign course with little or no disability after 10 or more years (Figure 1A). Progressive relapsing-remitting (Figure 1B) is characterized by progressive continuous disease from the outset. Secondary progressive MS (Figure 1C) is characterized by a steady worsening of symptoms. Most sufferers with relapsing-remitting MS will go on to develop this form of MS, usually after 5-15

years. About 10% of patients have primary progressive MS (Figure 1D) which is the most severe form, where the symptoms become steadily worse without relapses or remissions. Rarely, patients have malignant MS with a rapidly progressive course of disease.

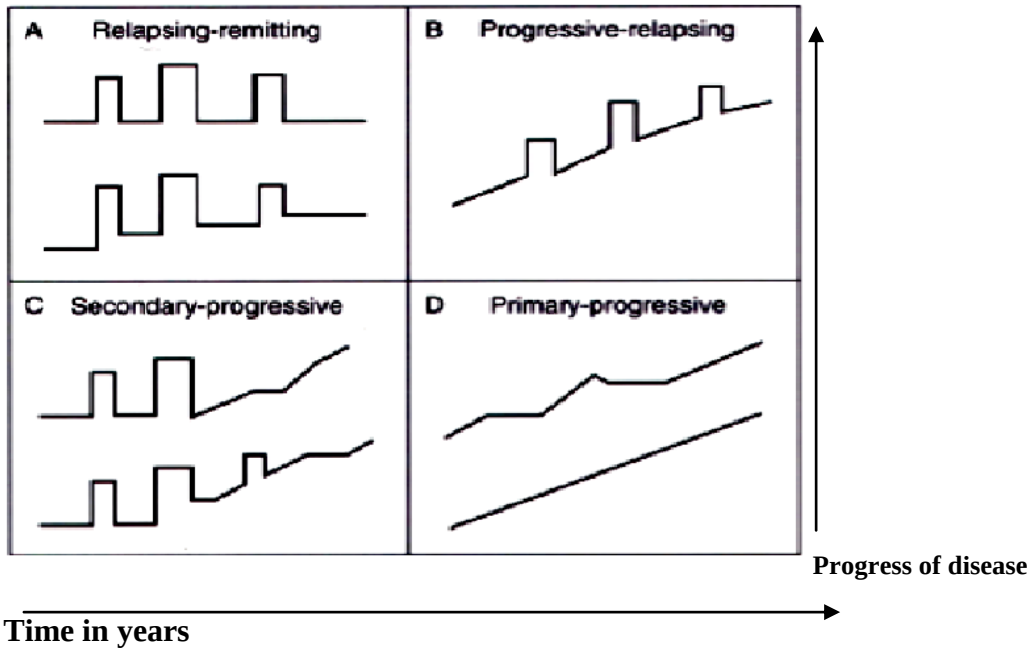

Figure 1.2. A representation of the four distinct clinical courses of multiple sclerosis. Figures show the progress of disease over time. (Whitaker and Mitchell, 1997). Figure (A) relapsing-remitting is characterized by alternating disease relapses and periods of full recovery or sequelae. Figure (B) progressive-relapsing is characterized by continous disease which is progressive from the onset. Figure (C) secondary-progressive presents as a relapsing-remitting disease but converts to a progressive course at a later time point. Figure (D) primary-progresses from onset, but has no definite acute relapses with or without remission.

MS is defined by the formation of lesions known as plaques, defined areas of demyelination seen in the white and grey matter of the brain. In normal brain tissue the neuronal axons are wrapped in myelin sheaths made from layers of cell membrane which are manufactured by oligodendrocytes, but in MS the myelin sheaths are stripped and axons degenerate. Phagocytic macrophages dominate

these inflammatory lesions. Demyelination results in the slowing of electrical impulses along the nerves, resulting in the focal neurological deficits that characterise MS. Demyelinated lesions can be found throughout the CNS but certain areas seem more vulnerable: the optic nerve, brainstem, spinal cord and periventricular regions, with the involvement of these regions often being symmetrical (Ffrench-Constant, 1994, pp. 271-275).

1.1.3 Epidemiology

Exhaustive epidemiological studies of MS have been conducted (Acheson, 1985, pp. 3-27; Brody 1972, pp. 173-176) and the fundamental question of whether the risk of contracting the disease is determined primarily by environmental factors or by genetics has repeatedly been asked. The answer has not been forthcoming, although it has been established that MS is not inherited as a single gene disorder (Compston, 1986, pp. 56-73; Roberts & Bates, 1982, pp. 287-293).

1.1.3.1 Genetics and racial prevalence

Studies have shown that there is only 28% concordance for MS amongst monozygotic twins and 2.5% amongst dizygotic twins (Ebers et al. 1986, pp. 1638-1642), indicating that genetic inheritance is not the major determinant and pointing to an important environmental factor in disease susceptibility. This was further highlighted by a recent study of monozygotic twins discordant for MS (Barazini et al., 2010, pp. 1351-1356). The researchers probed for differences in the genomes of three sets of monozygotic twins using DNA from their T cells and found no evidence of genetic, epigenetic or transcriptome differences to explain the disease discordance. Studies into adopted siblings and half siblings were performed by Dyment, Ebers and Sadovick (2004, pp.104-

110) to investigate if an environmental factor, such as a viral infection, may be a risk factor for MS, but there was no increased incidence in these groups.

There appears to be racial differences in susceptibility to MS with it being rare in Hungarian gypsies, Huterites, Eskimoes and Orientals, whilst there is a high incidence in people of Fennoscandian origin (Ebers & Bulman, 1986, p. 108). Northern Europeans are associated with Human leucocyte antigen (HLA) A3, B7, DW2 and DR2 (Batchelor, Compston & McDonald, 1978, pp. 279-284) but the strengths of the association with MS are low, with the relative risks being in the region of 5.0 compared with 87.8 with HLA B27 and ankylosing spondolytis (Gay, 1991).

Recent human genetic research into MS has found little evidence of a correlation with the disease. The strongest association has been found with the HLA-DR genotype, particularly HLA-DRB1, which is present in northern Europeans but not in people of African descent, where MS is rare (Oksenberg et al., 2004, pp.160-167; Ramagopalan & Ebers, 2008, pp. 283-285). Yet Hungarian gypsies have a high frequency of HLA-DR, but a low prevalence for MS (Gyodi et al., 1981, pp. 1-12) and so a clear genetic marker of susceptibility to MS has yet to be detected.

1.1.3.2 Geographical prevalence and migration

Although there appears to be a global distribution of MS (Ebers & Sadovnick, 1993, pp. 1-5) an association of MS prevalence with increasing latitude (Figure 1.3) was noted by Limburg (1950, pp. 15-24) and has been confirmed by many studies (Hammond, English & McLeod, 2000, pp. 968-974; Miller, Hammond, McLeod, Pudie & Skegg, 1990, pp. 903-905; Wallin, Page & Kurtzke, 2004, pp.

65-71). Dean (1967, pp. 724-730) showed that this cannot be accounted for by racial distribution when he found markedly lower rates of MS in decendents of white European populations in South Africa compared to the ancestral populations in England and Holland. This was confirmed by Miller and co-workers in a study of Australia and New Zealand, (Miller et al., 1990, pp.903-905) where they also found no correlation of MS with HLA-DR2 frequency. In a review of this data, Compston (1990, pp. 821-823) observed that the highest prevalence rates for these antipodean communities, with ancestry primarily from the United Kingdom (UK), was half the frequency found currently in the UK. He concluded that the antipodean environment must have some sort of protective effect.

Migration from a high risk (high latitude) to a low risk area results in a rate of MS intermediate between the native and host country (Baum & Rothschild, 1981, pp. 420-428; McCall, Sutherland & Acheson, 1969, pp. 151-165; Sutherland, Tyrer, Eadie, Casey & Kurland, 1966, pp. 57-67), unless the individual migrates after the age of 15 when they carry their risk with them (Alter, Kahana & Loewenson, 1978, pp. 1089-1093; Alter, Leibowitz & Speer, 1966, 234-237; Alter, Okihiro, Rowley & Morris, 1971, pp. 122-130; Dean & Elian, 1997, pp. 565-568; Dean & Kurtzke, 1971, pp. 725-729; Detels et al., 1978, pp. 386-393). Furthermore, it was found that children of Indian, African and West Indian immigrants to England have a similar incidence of MS as the indigenous English population (Elian, Nightingale & Dean, 1990, pp. 906-911). The epidemiological evidence therefore points to an environmental factor, possibly an infective agent acquired during early adolescence that leads to symptomatic disease after a lengthy latency period.

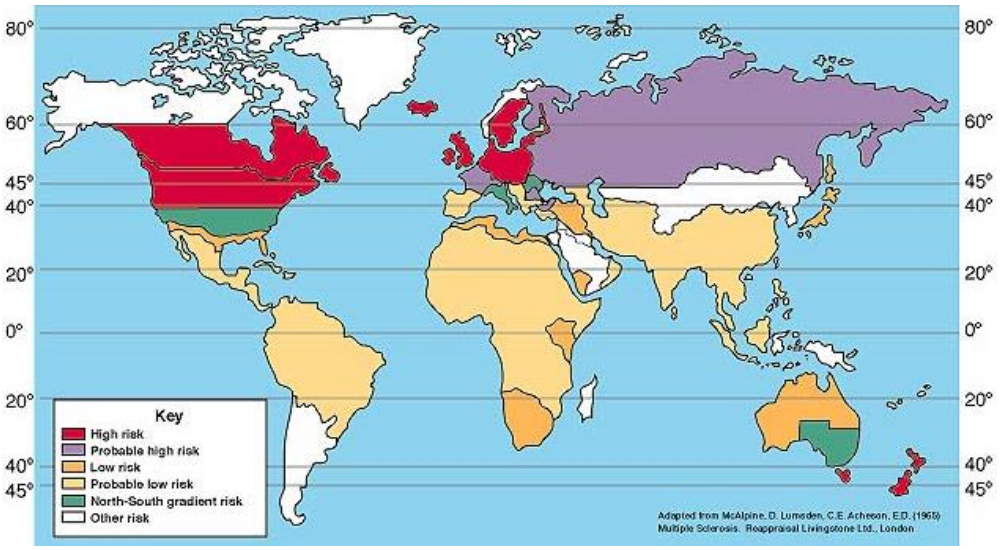

Figure 1.3. Worldwide distribution of multiple sclerosis as of 1994. **(**Kurtzke, 1997). Other risk = no data at present. This world pattern has been attributed to latitude and not migrational effects (Miller et al., 1990).

1.1.3.3 Epidemic multiple sclerosis

Kurtzke and Hyllested (1975, pp. 213-215; 1988, pp. 190-227) reported three consecutive decreasing epidemics of MS in the Faroe Islands, affecting native Faroese in the age group 11 to 45 years, from 1943 to 1973. MS had not apparently been recorded prior to 1943, and they attributed the epidemics to the transmission of an infectious agent by occupying British troops during World War II. Similar occurrences of epidemic MS were detected by Kurtzke (1993, p. 405) and by other researchers (Cook, Gudmundsson, Benedikz & Dowling, 1980, pp. 244-251; Cook et al., 1985, pp. 545-551) in Iceland, Shetland and the Orkney Islands. However, these observations have since been challenged by Poser who attributed 'epidemics' to problems with case ascertainment in these relatively isolated communities (Poser, Benedikz & Hibberd, 1992, pp. 143-152).

1.1.4 Prospective aetiologies for multiple sclerosis

1.1.4.1 Autoimmune hypothesis

Since the Second World War the theory that the demyelination seen in MS is due to an autoimmune T-cell mediated mechanism, although never proven, has through sheer repetition over the years become accepted as fact (McFarland & Martin, 2007, pp. 913-919). Indeed all mainstream modern therapies are based on the assumption that this hypothesis is correct (Compston & Coles, 2002, pp. 1221-1231). Reservations about this hypothesis occasionally emerge in the literature and have been heightened by the results of a survey of German, Hungarian and Polish MS patients (Greve, et al., 2008, pp.153-158). The researchers failed to find polymorphisms in the CTLA4 gene region, where a single nucleotide polymorphism CT60 is associated with many autoimmune diseases such as Grave's disease and autoimmune diabetes. Barcellos controversially claimed a higher frequency of autoantibodies in families with MS (Barcellos et al., 2006, pp. 924-931) but Ramagopalan was unable to confirm the findings (Ramagopalan et al., 2007, pp. 604-610), and a recent study by Qui and colleagues (Qui et al., 2010, pp. 147-155) failed to find any correlation between auto-antibodies and MS.

1.1.4.2 Viral hypothesis

The epidemiology of MS indicates that there is an important environmental trigger (Acheson, 1985, pp. 3-87) which could be microbial in origin. The search has focused on viruses, because of their well recorded latency. It has been proposed that the virus acts through molecular mimicry and induces an

autoimmune response from the host leading to demyelination (Acheson, 1985, pp. 3-87; Wucherpfennig & Strominger, 1995, pp. 695-705).

Molecular evidence of a straightforward antibody reaction to a viral antigen was gained by Owens and colleagues (Owens et al., 1998, pp. 236-243) but nothing has come of this research. More recently an Italian study claimed to have found evidence of Epstein Barr Virus (EBV) in MS brain tissue (Serafini et al., 2007, pp. 2899-2912) and in another study EBV was also implicated in MS (Pender, 2011, pp. 351-367) but these results have not been confirmed in other studies on EBV in MS (Hilton, Love, Fletcher & Pringle, 1994, pp. 975-976; Martin, 1997, pp. 280-283; Sargsyan et al, 2010, pp. 1127-1135; Willis et al, 2009, pp. 3318-3328).

1.1.4.3 Heterogeneity hypothesis

By the beginning of the 21st century the inability of researchers to establish a single cause of MS has led to the heterogeneity hypothesis (Lucchinetti et al., 2000, pp. 707-717). This claims that there are four distinct patterns of demyelination found in active demyelinating lesions in MS, and the researchers hypothesized that the disease consisted of four distinct diseases characterised by heterogenous pathogenetic mechanisms. In other words, since evidence could not be found that either of the two favoured aetiological disease processes, autoimmunity or viral infection, was the cause of all cases of MS, then both could be responsible, depending upon certain unknown factors. This idea gained considerable impetus due to the discovery that Devic's disease, or neuromyelitis optica (NMO), formerly classified as a variant of MS, was in fact a separate autoimmune disease (Lennon et al., 2004, pp. 2106-2112). Heterogeneity has

since been challenged by a Dutch team (Breiji et al., 2008, pp. 16-25). They found that there is uniformity in the active demyelinating lesions found in MS, and that any heterogeneity found in early lesions disappeared over time leaving an antibody and complement mediated myelin phagocytosis as the dominant general mechanism of demyelination. Any heterogeneity is therefore found within cases and not between cases. The problem with the heterogeneity hypothesis is nicely reviewed by Esiri (2009, pp. 39-41). She concludes that further evidence is required to justify the claim that MS is other than a single distinct disease.

1.1.4.4 Spirochaetal hypothesis

Pierre Marie, a pupil of Charcot who succeeded him in the Chair of Neurology at the Salpêtrière Hospital suggested in 1884 that MS might be caused by an infectious agent (Lamer, 1986, pp. 412-417). The search proved fruitless until the beginning of the next century when similarities between MS and known spirochaetal diseases led to the hypothesis, popular between 1909 and the 1950s, that MS might be caused by these microbes. The driving force for this hypothesis came from Germany (Schroeder, 1924, p. 785; Speer, 1921, p. 425) where spirocheatal organisms had been seen in central nervous system (CNS) tissue using silver stains. The advent of Nazism and the cataclysm of World War II resulted in the disappearance of both researchers and their work after 1935. Gabriel Steiner, one of the few researchers who remained, emigrated to America after the war and continued his work on spirochaetes until 1954 (Steiner, 1952, pp. 342-372). Acceptance of the spirochaetal hypothesis continued until 1957 when Ichelson (1957, pp. 57-58) published an ill-fated paper in support of the

spirochaetal theory, only then to retract her findings admitting that her distilled water had been contaminated with spirochaetal organisms. The long-term damage was devastating for spirochaetal research, with the influential MS Society refusing to fund further work and thus discouraging other bodies from supporting research, even to the present day (Gay & Dick, 1986, pp. 75-77).

1.1.4.5 Chlamydial hypothesis

Despite the great advances made in clinical microbiology since Steiner's work, no infective agent has been found. It was thought that *Chlamydia pneumoniae* was implicated in MS development (Sriram, Mitchell & Stratton, 1998, pp. 571-572) but this has since been challenged by the findings of a number of workers (Bowman, Roblin, Sundstrom & Hammerschlag, 2000, p. 265; Furrows et al., 2004, pp. 152-155; Hammerschlag et al., 2000, pp. 4274-4276).

1.2 Bacteria, sinusitis and multiple sclerosis

1.2.1 Oligoclonal bands

In the 1940s it was discovered that the cerebrospinal fluid (CSF) of MS patients contained an increase in gamma globulins which on electrophoresis show highly restricted banding (Kabat, Moore & Landow, 1942, pp. 571-577). These oligoclonal bands of IgG are found in high concentrations in greater than 95% of patients presenting with MS suggesting an inflammatory immune response to a foreign antigen. They are, however, not normally found in the *serum* of MS patients, suggesting that an antigen has entered the central nervous system (CNS) provoking a B-cell mediated immunological reaction. Oligoclonal bands are one

of the few consistent and observable features of MS, and although they are used as a disease marker the reason for their production remains a mystery. They are not specific for myelin, or other "self" antigens, and appear to be unrelated to micro-organisms (F.W. Gay, 2007, pp. 105-112). However, iso-electric focusing of MS CSF followed by immuno-blotting with *Staphylococcus aureus* antigens resulted in evidence that some of the oligoclonal antibodies were *S. aureus* specific (Jorge, 1995).

1.2.2 Multiple sclerosis, optic neuritis and sinusitis

Optic neuritis arises from an inflammation, and accompanying demyelination, of the optic nerve. This results in blurred vision, loss of visual acuity, dyschromatopsia, complete or partial blindness, and pain behind the eye. It usually appears unilaterally, and in the 15-50 year age group it is the most frequently presenting symptom of the onset of MS (Hutchinson, 1976, pp. 283-289; McAlpine, 1972, pp. 148-163).

The idea that optic neuritis was in some way linked to paranasal sinusitis appeared in the literature throughout the 18th and 19th centuries (White, 1917, pp. 891-899), and in the early 20th century a series of reports led to the belief that infections in the posterior ethmoidal and sphenoidal air sacs led to optic neuritis (Crane, 1927, pp. 201-240).

By the end of the 1920s a considerable body of evidence had built up linking optic neuritis and sinus disease (Thomson, 1929, pp. 248-261). When Herzog (1928, pp. 292-321) histologically examined twelve cadavers he discovered considerable variation in the structure and density of the bone separating sphenoidal and ethmoidal cells from the optic canal. Reviewing sinus tissue

from cases of optic neuritis he observed that even where the epithelium appeared to be healthy the mucosa showed evidence of chronic inflammatory degeneration. Oliver and Crowe (1927, pp. 503-525) and McMahon (1926, pp. 310-333) corroborated his findings. It became apparent that the structure of the bony wall of the optic canal was an important determinant of the outcome of any mucosal infection. These findings were especially interesting when added to the evidence of anatomical research performed by Onodi (1908, pp. 1-61) and Van Alyea (1941, pp. 225-253). They found that during the second decade of life excavation of the sphenoid bone led to the formation of recesses which penetrated the middle cranial fossa. This resulted in the optic nerve meninges being separated from the sinuses by thin layers of bone through which the sinus mucosa sometimes herniated, leading Onodi (1908, pp. 1-61) to speculate that a pathway might be opened for chronic infections of the sinuses to extend into the CNS (Figures 1.4 and 1.5). Kramer and Som (1940, pp. 744-770) were later to demonstrate this in cases of 'cryptogenic' bacterial meningitis.

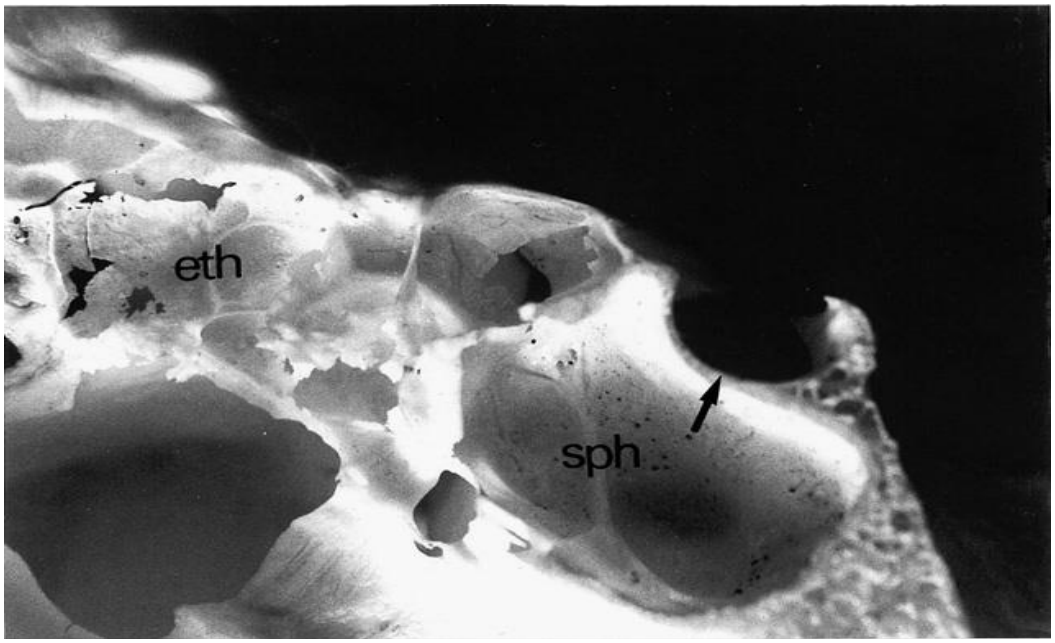

Figure 1.4. Median sagittal section through the sella turcica (arrow) showing deeply penetrating sphenoidal (sph) and ethmoidal (eth) cells, with paper thin walls. Onodi collection. Royal College of Surgeons of England.

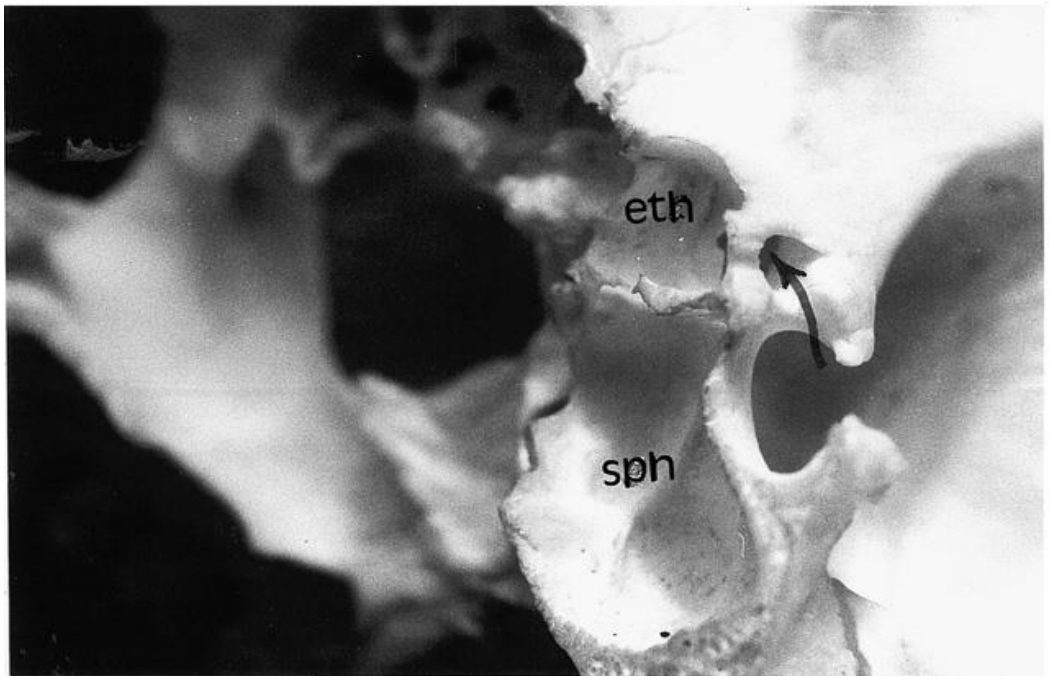

Figure 1.5. Localised defect in the wall of the optic canal (arrow), communicating with spheno-ethmoidal sinus. Onodi collection. Royal College of Surgeons of England.

Harris Vail (1931, pp. 846-863) using X-rays and surgical exploration implicated posterior sinus infection as a cause of optic neuritis. He also made the observation that optic neuritis, like sinusitis, was commoner in the colder months of the year, with patients often recalling having had coryza or 'flu' prior to the attack. In 1926 McMahon (pp. 310-333) established that spheno-ethmoiditis was twice as common in females, with a steep rise in the third decade of life followed by a gradual decline in the fourth and fifth decades. This seemed to show that spheno-ethmoiditis and optic neuritis were clinically related and shared distinctive epidemiological features.

Derek Vail in 1938 (pp. 383-394) suggested that the posterior sinuses might form a portal through which toxins or viruses could enter, such that MS, optic neuritis and optochiasmatic arachnoiditis might be part of the same pathological process. During the period between the world wars it was perceived that surgical drainage of the posterior sinuses relieved the condition of optic neuritis, even though Brunner (1941, pp. 903-929) pointed out that many cases of optic neuritis resolved spontaneously if left alone (F.W. Gay, 2007, pp.105-112). It was found that in most instances optic neuritis led to MS, with figures as high as 75% in temperate latitudes (Shibasaki, McDonald & Kuroiway, 1981, pp. 253-271). It was thus surmised (incorrectly) that since optic neuritis was caused by MS, it could not be caused by sinusitis. The implication was that the cause of MS was known, with sinusitis being excluded as a possible cause.

1.2.3 Evidence for a link between sinusitis and multiple sclerosis

There are a number of clinical and epidemiological similarities between these two diseases. Both are chronic diseases characterised by exacerbations and

remissions. Both MS and sinusitis have been shown to be activated by minor viral respiratory infections (Anderson, Lygner, Bergstrom, Anderson & Vahlne, 1993, pp. 417-422; Johnson, 1994, pp. S54-S60; Panitch, 1994, pp. S25-S28; Sibley, Bamford & Clark, 1984, pp. 14-24).

One of the most distinctive features of MS is the age attack distribution which shows few cases before the age of 15 years with rates rising rapidly to the age of 30 years and then declining again with relatively few cases after the age of 35 years. Despite geographical differences in incidence, this age-attack curve remains remarkably constant and both Brody (1972, pp. 3-27) and Acheson (1985, pp. 173-176) contended that it was unlike any contagious disease known. In 1986, an association between MS and chronic sinusitis, with the peak in sinus infection occurring in the year immediately preceding the first recorded episode of MS, was noted by Gay, Dick and Upton (1986, pp. 815-819). It was also noted that sinusitis, like MS, showed a tendency to affect females more frequently than males (a ratio of 2:1) and at an earlier age (Gay, Dick & Upton, 1986, pp. 815-819, Callaghan, 1986, p. 160). Both MS and sinusitis are seasonal, showing a peak of attacks that coincides in the spring and summer in both the northern and southern hemispheres (Auer, Schumann, Kumpfel, Gossi & Trenkwalder, 2000, pp. 276-278; Callaghan, 1986, p. 160-161; Gay, Dick & Upton, 1986, pp. 815-819; Salvi et al., 2010, p. 105; Sibley, Bamford & Kent, 1983, pp. 14-24; Tremlett, et al., 2008, pp. 271-279). The incidences of both diseases increases with increasing latitude, possibly indicating a relationship to climate (Acheson, 1985, pp. 3-46; Gunderson & Hävag, 1971, pp. 939-943), and both show the carriage of risk, fixed during the later teenage years, on migration from high to low incidence locations. In chronic sinusitis this is linked to

continuing mucosal damage to cells caused by repeated attacks of low grade infection (F.W. Gay, 2007, pp. 105-112). Since the sphenoidal sinuses undergo their major development post puberty, mucosal damage of these sinuses by microbes would be limited in children, and if MS and chronic sinusitis are linked this would also explain the onset of MS after puberty (Dick and Gay, 1988, pp. 25-35). Furthermore, if there is an association between the two diseases then a person migrating from a high-risk area before the age of 15 years to a warm climate where the risk of MS is low (Martin, 1967, pp. 1079-1098), would avoid the risk of chronic sinus damage, and therefore would not carry the associated high risk of MS. In 1988 Dick and Gay (pp. 25-35) postulated that a microbe or its products could enter the CNS *via* the posterior paranasal sinuses in MS patients, but since then nose to brain transmission of transportable toxins released into the mucosal tissues by bacterial flora in the paranasal sinuses, has received little attention. Hawkes and co-workers (Hawkes, Shepherd & Daniel, 1999, pp. 473-480) noted the impaired sense of smell in a number of neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease. Impairment of olfactory function is also a feature of early active MS (Lutterotti et al., 2011, pp. 964-969) and could be indicative of a connection between the nose and brain.

1.2.4 Bacterial infections and multiple sclerosis

Whilst the search for viruses continues, bacterial causes of MS have been largely ignored. This omission is puzzling as bacteria are known to produce an array of products and have been implicated in 'autoimmune' molecular mimicry (Moran, Prendergast & Appelmelk, 1996, pp. 105-115). In recent years many diseases with previously unknown aetiologies have been shown to be caused by bacteria.

These include Legionnaire disease (McDade et al., 1977, pp. 1197-1203), haemolytic-uremic syndrome (Griffin & Tauxe, 1991, pp. 60-98) and Lyme Disease (Steere et al., 1983, pp. 733-740). The dogmatic assumption of a viral aetiology for MS was summed up by Marshall (1988, pp. 89-92) as consisting of the argument "MS is a viral disease, we do not know which virus yet we will find the right one someday." An example of the dangers of such tunnel vision can be seen with gastritis and acid-peptic ulceration. Generations of physicians were taught that the extremely acidic environment of the stomach meant that bacteria could not survive there, so that although bacteria were seen in gastric acid-peptic ulcer biopsies they were cursorily dismissed as transient contaminants. Even when Marshall and Warren (1984, pp. 1311-1315) presented evidence of *Helicobacter pylori* as the cause of chronic ulcerative gastritis they were ignored or disbelieved for a decade until the evidence, largely due to the efficacy of antibiotics, eventually proved irrefutable.

A review by Trapp (2004, pp. 455-457) on the lack of progress in establishing the pathogenesis of MS states that there is a 'missing link' in the understanding of MS. If this 'missing link' is the presence and processing of microbial antigens then bacterial products provide the best fit. Bacteria produce transportable antigens such as β toxin (sphingomyelinase) which are complement-fixing and toxic for oligodendrocytes, resulting in cell apoptosis (Esen et al., 2011, pp. 431-439). Reports of the presence of Gram positive cell wall lipotechoic acid in MS plaque macrophages (Schrijver et al., 2001, pp. 1544-1554; Visser et al., 2006, pp. 1671-1685) suggest that bacterial antigens may be involved, especially as peptidoglycans have been implicated in chronic inflammation (Dziarski, 2003, pp. 1793-1804).

There have been some studies performed to investigate if bacterial infections can initiate relapses of MS (Sibley, Bamford & Clark, 1985, pp. 1313-1315; Buljevac, et al., 2002, pp. 952-960) but more have been performed on experimental autoimmune encephalomyelitis (EAE), the animal model for MS. Enterotoxins from *Staphylococcus aureus*, which can act as T-cell activating superantigens, can exacerbate or induce EAE (Matsumoto & Fujiwara, 1993, pp. 268-278; Schiffenbauer, Johnson, Butfiloski, Wegrzyn & Soos, 1993, pp. 8543-8546; Soos, Hobeika, Butfiloski, Schiffenbauer & Johnson, 1995, pp. 6082-6086; Soos, Mujtaba, Schiffenbauer, Torres & Johnson, 2002, pp. 30-34). Additionally a worsening of EAE in mice infected with *Streptococcus pneumoniae* has been reported (Herrman et al., 2006, pp. 4841-4848). This ability of pathogens to influence the course of autoimmune diseases is due to their intrinsic activation of Toll-like receptors (TLRs) which recognise specific patterns of microbial components and regulate the activation of innate and adaptive immunity (Prinz et al., 2003, pp. 195-199; Takeda & Akira, 2005, pp. 1-14). Bacterial lipopolysaccharides are potential modifiers of autoimmune inflammation (Hamada, Driscoll, Kies, & Alvord, 1989, pp. 275-284; Segal, Chang & Shevach, 2000, pp. 5683-5688; Segal, Klinman & Shevach, 1997, pp. 5087-5090, Visser et al., 2005, pp. 808-816) and are able to activate the innate immune system via specialized receptors such as TLRs situated on antigen presenting cells (Takeda & Akira, 2005, pp. 1-14). Although there is little data on Gram positive bacteria modulating EAE, exacerbation of MS after active immunization with a pneumococcal vaccine has been reported (Tan, 2002, pp. 3-9). Herrmann et al. (2006, pp. 4841-4848) have postulated that this exacerbation is Toll-like receptor 2 (TLR2) dependent but T cell independent, and speculated

that the action of *S. pneumoniae* or other bacteria on the immune system in MS is greatest in the subclinical phase of infection and occurs prior to the manifestation of an MS event.

1.2.5 Possible mechanisms for a bacterial aetiology

If it is accepted, then, that there could be a bacterial cause of MS, why has the organism not yet been identified? There are several reasons why this could be. The bacteria could be fastidious organisms that will not grow on conventional bacteriological culture media. Bacteria which are now well recognized as human pathogens such as *Campylobacter jejuni* (Dekeyser, Goussuin-Detrain, Butzler & Sternon, 1972, pp. 390-392) and *Legionella pneumophila* (McDade et al., 1993, pp. 1197-1203), and more recently *Helicobacter heilmannii* (Sykora, Hejda, Stozicky, Siala & Schwarz, 2004, pp. 707-709) were not identified as causes of human disease until specialized techniques were employed. Similarly, the organism could fail to be isolated because of a slow growth rate, as happened with *Bartonella henselae* the cause of cat scratch fever (Zangwill et al., 1993, pp. 8-13) bacillary angiomatosis (Koehler, Quinn, Berger, LeBoit & Tappero, 1992, pp. 1625-1631) and with submasseteric abscesses with *Mycoplasma salivarium*
(Grisold et al., 2008, pp. 3860-3862).

Conversely the organism may be relatively easily grown but located in a region of the body with a large and complex normal flora. Such pathogens usually exert their effect by toxin production such as *Escherichia coli* O157:H7, which was not routinely looked for as a cause of food poisoning until clusters of bloody diarrhoea led to its recognition as a pathogen (Besser et al., 1993, pp. 2217-2220)

and more recently *Escherichia coli* O104: H4, which produces a Shiga-like toxin (Muniesa, Hammerl, Hertwig, Appel & Brussow, 2012, pp. 4065-4073).

The bacterial pathogen potentially causing the disease may also do so as the result of uncommon sequelae of common infections. The effects may be distant in both location and time. *Campylobacter* enteritis can initiate Guillain-Barré syndrome in 1 case out of 2000 infections (Mishu & Blaser, 1993, pp. 104-108). Blaser speculated as long ago as 1994 (pp. 144-145) that there is plenty of evidence of bacterial causes of chronic inflammatory disease, and if a demyelinating disease such as Guillain-Barré syndrome can be triggered by a bacterial infection (Hughes & Cornblath, 2005, pp. 1653-1666) then why not MS? Speculation of a bacterial aetiology has foundered on the inability to isolate an organism by bacterial culture, and this was thought by advocates of this hypothesis to be due to technological shortcomings. The development of molecular techniques, especially using primers of the 16S rRNA genes of bacteria, gave fresh impetus to researchers looking to discover bacterial causes for diseases. The use of universal primers to investigate diseases of unknown aetiology such as MS was an attractive proposition (Grieson, Loeffelholz, Purohit & Leong, 1994, pp. 335-351; Relman, Schmidt, MacDermott & Falkow, 1992, pp. 293-301). Universal primers rely on the fact that some areas of the 16S rDNA are conserved among all bacteria, while some are conserved within a genus or a larger group, as well as there being areas unique to individual bacterial species. The use of universal primers to amplify all bacteria is attractive in practice but is useful only for those specimens with a relatively high bacterial concentration. Bacteria and their DNA are ubiquitous so that there is a background level of bacterial DNA contamination. Lindsey and Patel (2008, pp.

147-152) estimated this background level of contamination in their laboratory to be 200 genomes per reaction in their laboratory and found that the CSF of MS patients in their study was never higher than this level. After sequencing the PCR products, they found that the most common contaminants were uncultured species of *Pseudomonas* and *E. coli*. Other researchers also encountered contamination problems when using universal primers (Corless, Guiver, Borrow, Edwards-Jones, Kaczmarski & Fox, 2000, pp. 1747-1752; Grieson, Loeffelholz, Purohit & Leong, 1994, pp. 335-351). Semi-universal primers, using a nested PCR, have been used to examine MS CSF (Lindsey & Patel, 2008, pp. 147-152) but despite the increased sensitivity of this technique no DNA of spirochaetes, *Campylobacter, Mycoplasma, Chlamydia, Bartonella, Mycobacteria* or *Streptococcus* were found. Lindsey and Patel did not rule out a bacterial aetiology for MS, but concluded that until they had an idea of what the causative organism was likely to be, such an approach was unlikely to be successful as the number of potential candidates was so large.

Before Steiner (1952, pp. 343-372) proposed his spirochaetal hypothesis of MS, Dawson (1916, pp. 517-740) had hypothesized that bacterial toxins intermittently disseminated in the CSF could damage the blood brain barrier and result in perivascular demyelination, and Vail (1938, pp. 383-394) suggested that bacterial toxins could diffuse into the CNS via lesions in the paranasal sinuses.

Gadolinium-enhanced nuclear magnetic resonance imaging showed that foci of blood-brain barrier leakage were the earliest detectable change in the central nervous system in MS (Grossman et al., 1988, pp. 117-122).

The most compelling model put forward though is by Gay (F.W. Gay, 2007, pp. 105-112). This bacterial toxins hypothesis postulates that an intermittent leakage

of a mixture of bacterial toxins from the posterior paranasal sinuses into the CNS results in MS. As already detailed in section 1.2.3 there are marked similarities between sinusitis and MS, and Gay's hypothesis links the two conditions.

1.3 The bacterial toxins hypothesis

1.3.1 Association of bacterial toxins with sinusitis and multiple sclerosis

As mentioned in section 1.2.3 there are marked similarities between sinusitis and MS, and the possibility of a link between the two diseases. Anatomical and physiological studies show that the posterior paranasal sinuses lie close to the optic nerve meninges and the contiguous structures of the spinal cord and brain. Furthermore the sub-mucosa of cells in the ethmoidal and sphenoidal sinuses often lie on the optic nerve and adjoining basal dura without the protection of intervening bone (F.W. Gay, 2007, pp. 105-112). Cranial nerves, blood vessels and lymphatics also pass through the structures of this region thus providing an opportunity for the spread of bacterial products from the sinuses into the CNS (Esiri & Gay, 1990, pp. 3-8; Foldi, 1977, pp.121-124; Rennels, Gregory, Blaumanis, Fijimoto & Grady, 1985 pp. 47-63). Studies have shown that both spinal and cranial nerve sheaths are able to carry bacterial toxins centrally to the CNS (Orr & Rose, 1914, pp. 271-340; Wright, 1953, p. 319).

1.3.2 The blood-brain barrier

It has long been known that there is a blood-brain barrier which shields the CNS from the immune system. This 'immunological privilege' (Billingham &

Boswell, 1953, pp. 392-406) was thought to be due to the CNS lacking a lymphatic drainage system. However, it is now well established that a variety of drugs, some possessing molecules too large to pass the blood-brain barrier rapidly access the CNS when administered intranasally (Hanson & Frey, 2008, pp. 1-6). It has become clear that there are channels surrounding the cranial nerves and the cerebral blood vessels that provide a bi-directional flow between the CNS tissues and the local lymphatic system (Becher, Prat & Antel, 2000, pp. 293-304; Esiri & Gay, 1990, pp. 3-8; Rennels et al., 1985, pp. 47-63). Protein tracers have shown that there are bidirectional links beween the extracellular fluid compartment of the CNS and the lymphatic system of the head and neck via sheaths in the meningeal vessels and perineural cranial nerves (Esiri & Gay, 1990, pp. 3-8; Johansson, 1995, pp. 1-19; Rennels et al., 1985, pp. 47-63). These channels permit the CNS to mount an immunological response to antigens within the CNS tissues. This means that it is possible for antigens being processed in the mucosa of the nasopharynx to drain into the CNS and be processed by the immune cells in the meninges and perivascular space. Toxins released by microbes in the sinuses can therefore enter the CNS and gain access to the brain and spinal cord (F.W. Gay, 2007, pp. 105-112). The inflammation seen in MS in the trans-cisternal trigeminal nerve that was observed by Orr and Rose in 1914 (Orr & Rose, 1914, pp. 271-340) may therefore be the result of ascending lymphatic drainage from the nasopharynx. The clinical, epidemiological and pathogenic similarities between MS and nasopharyngeal sinusitis is thus consistent with the bacterial toxins hypothesis (F.W. Gay, 2007, pp. 105-112).
It has been realised for some time that in MS apparantly normal myelinated CNS tissue located some distance from plaques show significant abnormalities. Blood

vessels traversing myelinated white matter show inflammatory cell perivascular cuffing (Adams, 1975, pp. 165-182; Adams, 1989, pp. 130-139), and activated astrocytes (McKeown & Allen, 1978, pp. 471-482). McKeown and Allen (1978. pp. 471-482) postulated that these changes represented the primary pre-demyelinating process. In immunocytochemical studies using early post-mortem tissue (Barnett & Prineas, 2004, pp. 458-468; Gay, 2006; pp. 234-240; Gay, Drye, Dick & Esiri, 1997, pp. 1461-1483; Groome, Hayes, Woodroofe, Glynn & Cuzner, 1987, pp. 463-467; Li, Cuzner & Newcombe, 1996. pp. 207-213; Sanders, Conrad & Tourtellotte, 1993, pp. 207-216) areas of normal appearing white matter located in perivascular, subependymal and subpial parenchyma were populated by HLA DR+ microglia with clear morphological evidence of cellular activation. These lesions may represent primordial plaques as the microglia show evidence of containing myelin-peptide and basic protein (F.W. Gay, 2007, pp. 105-112). These 'activated' regions were found in the parenchyma and were virtually devoid of T-cells as well as showing no evidence of plasma-protein leakage suggesting an intact blood brain barrier (Esiri & Gay, 1997, pp. 173-186; Gay et al., 1997, pp. 1461-1483; Gay, 2006, pp. 234-240). Some lysis of myelin appears in this region but the opsonisation of the sheath with immunoglobulin and complement sometimes seen in plaques (Storch et al., 1998, pp. 465-471) is absent (Gay et al., 1997, pp. 1461-1483; Gay, 2006, pp. 234-240). In some areas of microglial activation, co-locating C3d and IgG were found on the microglial cell membranes suggesting the capture of immune complexes by these cells (Esiri & Gay, 1997, pp. 173-186; Gay et al., 1997, pp. 1461-1483; Gay, 2006, pp. 234-240). In these studies, C3d and IgG were also found on HLA DR+ macrophages in the Virchow Robin (VR) spaces of vessels

crossing normally myelinated tissues, as well as being found free on the glia limitans. This has led to speculation that extrinsic complement-fixing antigens, such as bacteria or bacterial toxins, are being processed in the VR spaces and an excess of the antigens could then spill over across the glia limitans into the surrounding parenchyma leading to a local activation of perivascular microglia and resulting in the iniation of myelin damage (Esiri & Gay, 1990, pp. 3-8; Gay, 2006, pp. 234-240).

1.3.3 Evidence for bacterial toxins in multiple sclerosis

Histological stains revealing erosion and lysis of myelin sheaths that were suggestive of a primary lytic factor were observed as long ago as 1955 (Lumsden, 1955, pp. 208-239). In experimental allergic encephalomyelitis (EAE), which is often employed as a model for MS, myelin loss is thought to be due to the 'bystander' effect caused by proteinases produced by T cells (Brosnan, Cammer, Norton & Bloom, 1980, pp. 235-237). Yet Raine (1997, pp. 243-286) has highlighted the problem of extending this hypothesis to MS, namely none of the chronic and inflammatory diseases of the CNS induce plaque formation. Barnet and Prineas came to the same conclusion and suggested that there was a specific pathogenic attack on the myelin (Barnett & Prineas, 2004, pp. 458-468). So what evidence is there for an extrinsic antigen, such as a bacterial toxin, being involved in the pathology of MS?

A powerful polyclonal B-cell response has been found in the CNS in MS with antibodies produced to a variety of microbial and other antigens (Walsh & Tortellotte, 1983, pp. 275-358) that is characteristic of the response to bacterial cell wall polymers (Räsänen & Arvilommi, 1981, pp. 712-717). This response is

accompanied by repeated bursts of oligoclonal antibody production that coincide with disease activity measured by computerised tomography (CT) and magnetic resonance imaging (MRI) scans (Sharief & Thompson, 1991, pp. 181-195). This is suggestive of stimulated B lymphocytes reacting with T cell independent antigens such as bacterial cell wall polymers. The random release of bacterial antigens into the CNS should result in changes of the oligoclonal banding pattern during the course of MS, something which Thompson, Kaufmann and Rudge (1983, pp. 547-550) have observed.

In 1991 small plaques in early MS were found to contain particulate material within macrophages and astrocytes, on which complement and immunoglobulins were co-located (Gay & Esiri, 1991, pp. 557-572) (Figure 1.6). These were absent in chronic MS, indicating that the inflammatory changes had a degree of specificity that could be related to the antigens within the macrophages and astrocytes. Further studies by Gay (2006, pp. 234-240) indicated the presence of a diffuse, complement-fixing antigen. This was located in the Virchow-Robin spaces. It was speculated that a bacterial lipase may be the antigen. Exogenous superantigens have been of interest in this context. These are a heterogeneous group of cellular and transportable molecules that result in the non-specific proliferation of T and B cells by binding to the Vβ region of the T cell receptor and the α chain of the major histocompatability complex (MHC) (Friedman, Posnett, Tumang, Cole & Crow, 1991, pp. 468-480). Although Rudge speculated that an unknown retrovirus could produce them in MS patients (Rudge, 1991, pp. 853-855) superantigens are produced by many Gram positive organisms, such as streptococci and staphylococci, and Kotb (Kotb, 1995, p. 416) speculated that bacterial superantigens may cause MS.

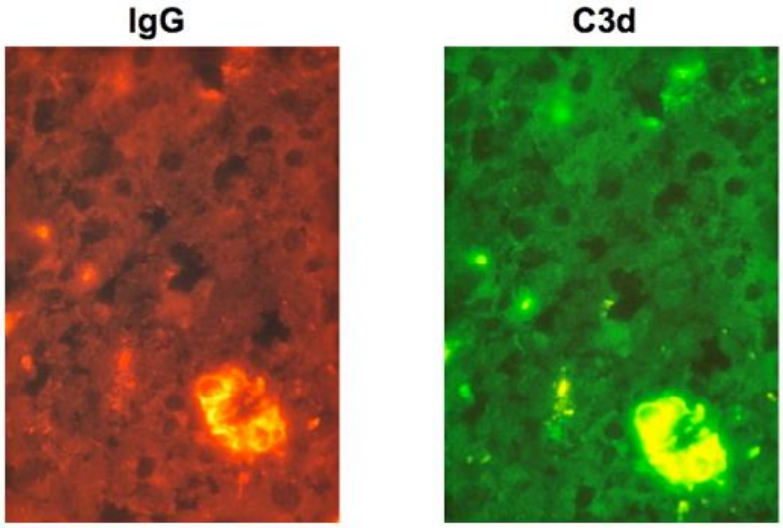

Figure 1.6. Immunocytochemistry of acute multiple sclerosis tissues. Co-locating IgG and C3d. Immune complexes are located on the membranes of activated macrophages. Anti-human IgG-TRITC; anti-human C3d-FITC (Gay, Drye, Dick & Esiri , 1997).

1.4 *Dolosigranulum pigrum*

1.4.1 Initial isolation and identification

Dolosigranulum pigrum was first isolated in 1988 at the Microbiology Department of Colchester General Hospital (Aguirre, Morrison, Cookson, Gay & Collins, 1993, pp. 608-612) from spinal cord tissues. The tissues were obtained at a coroners post mortem from a case of suicide (hanging) of a newly diagnosed case of MS, one week after onset of an acute attack of the disease. The tissue had been frozen in liquid nitrogen at post-mortem and then stored at -80°C, and was donated by the Institute of Neurology, National Hospital for Neurology and Neurosurgery, Queens Square, London WC1N 3BG to Dr FW Gay, who in 1988 was conducting research at the Colchester Hospital laboratory into the possible

role of bacterial infections in MS. Gay and co-workers (Gay et al., 1997, pp. 1461-1483) found groups of C3d-IgG coated 1.0-1.5 μm ovoid bodies (Figure 1.4) resembling bacterial cells scattered throughout the meningeal tissues of the spinal nerve roots, and in the phagocytic vacuoles of activated macrophages within MS lesions (Gay et al., 1997, pp. 1461-1483).

Although bacterial cells are found as post mortem contaminants, the presence of surface complement and immunoglobulin in this case suggested that the oval bodies (if bacterial in nature) had invoked an ante-mortem immune response. The fact that the tissue had been snap frozen during autopsy and then stored at -80°C raised the possibility that bacterial cells within tissue would remain viable, and so, in an attempt to identify the organism, cut surfaces of the frozen tissue blocks were impressed onto the surface of Columbia agar with 5% horse blood, nutrient agar and chocolate agar, and incubated at 37°C, aerobically, anaerobically and with 5% carbon dioxide. Micro-colonies of less than 0.5 mm were seen after 72 h around the periphery of the impressions on the aerobic blood and chocolate agar plates. Growth was also seen on the nutrient agar plates. Sub cultures were made on Columbia agar with 5% horse blood and incubated at 37°C. After 48 h, a faint growth was visible that after a further 24 h appeared as non-haemolytic dry, grey/white microcolonies less than 1.0 mm in diameter. After 5 days incubation the colonies were convex, white and glossy and 1.0 mm in diameter and demonstrated α haemolysis (Figure 1.7). There were tiny colonies formed that upon sub-culture resembled the main growth. The plates were then incubated for a further 3 days at an ambient temperature of 18°C and developed β haemolysis (Figure 1.8).

Figure 1.7. *Dolosigranulum pigrum* on aerobic Columbia blood agar showing α haemolysis after 5 days aerobic incubation at 37°C.

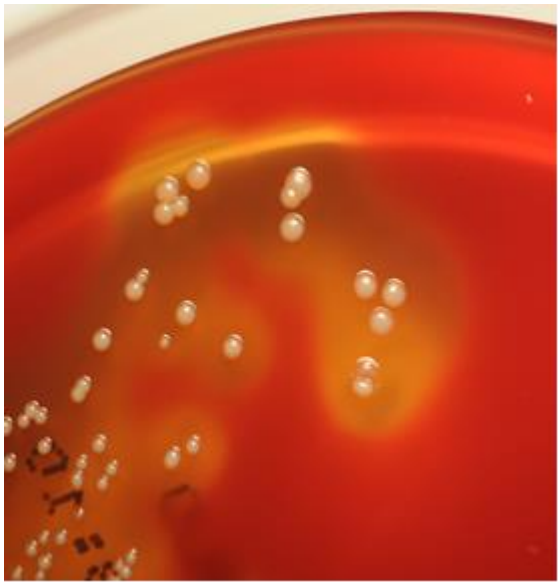

Figure 1.8. *Dolosigranulum pigrum* on aerobic Columbia blood agar showing β haemolysis after 8 days aerobic incubation at 37°C and 3 days at 18°C.

On two further occasions, using the same methods, the organism was isolated in pure culture and was sub-cultured for identification by the author (F.M. Jorge, November, 1989). The bacteria were catalase negative, Gram positive cocci, 1.0-1.5 μm in size, ovoid in shape and arranged in clumps. Using the API 20 STREP system (bioMerieux), positive reactions were seen with the pyrrolidonyl arylamidase, leucine aminopeptidase and arginine dihydrolase tests. The profile did not fit with any bacteria on the API database, but was closest to *Gemella haemolysans*. These findings were confirmed by API bioMerieux (UK) Limited, and by the Laboratory of Hospital Infection, Colindale, London.

1.4.2 Characterization and naming of *Dolosigranulum pigrum*

The bacterium was isolated again in 1991 at the RAF Institute of Pathology and Tropical Medicine, Halton, Aylesbury, from a patient with neurotropic cornea (Hall et al., 2001, pp. 1202-1203). A full phenotypic and phylogenetic characterization of the organism, using 16S rRNA DNA sequencing studies, was then carried out at the Department of Microbiology, Agriculture and Food Research Council, Reading. Phylogenetically the organism was closest in affinity to *Aerococcus* and *Globicatella* but, being genealogically distinct, was classified in a new genus, *Dolosigranulum*, of which it is the sole species (Aguirre et al., 1993, pp. 608-612). The original isolate from Colchester was deposited as the type strain at the National Collection of Industrial, Marine and Food Bacteria, Aberdeen (NCIMB 702975). It was named *D. pigrum* gen.nov.,sp nov. (Aguirre et al., 1993, pp. 608-612) by Professor Ozro MacAdoo at Virginia Polytechnic and State University, Blacksberg, Virginia, USA, (*dolosus*, Latin, crafty, deceitful; *granulum* a small grain, *pigrum*, Latin, lazy).

As the name suggests *D. pigrum* can be difficult to culture and detect due to the long incubation time and the initially small colonies it forms. It grows more readily on solid medium than in liquid culture, but liquid culture is possible using 'brain heart infusion' broth. Further studies have confirmed that the organism is catalase negative and although initially it demonstrates α haemolysis, incubation for more than a week at either 37°C or at room temperature or prolonged storage at 4°C, results in β haemolysis. It is possible, therefore, that *D. pigrum* produces β haemolysins as stress protein if growth is inhibited or restricted, but this remains to be investigated. The initial 16S rRNA sequencing yielded the *D. pigrum* gene sequence from the type strain (acc. X70907). By the beginning of the next century it was apparent that the bacterium was a significant pathogen with isolates being reported in specimens such as blood cultures and eye specimens (Table 1.1, LaClaire & Facklam, 2000a, pp. 2001-2003). Since then it has been implicated in a number of infections (section 1.3.5).

Table 1.1. Sources, clinical diagnoses, and demographic information on 27 strains of *Dolosigranulum pigrum* (LaClaire & Facklam, 2000).

Strain (CDC no.)	Source	Clinical diagnosis	Age	Sex	Locale
SS-1342	Spinal cord	Multiple sclerosis; autopsy			England
623-80	Eye		43 yr	M	S.Dak.
577-86	Sputum	Pneumonia		M	S.Dak.
2059-90	Eye	Blepharitis	74 yr	M	Nebr.
106-91	Blood		80 yr	F	Mich.
810-92	Blood	Sepsis	Adult		N.C.
1869-94	Eye		76 yr	F	N.Y.
3295-94	Blood	Sepsis	1.2 yr	F	N.C.
39-95	Sinus	Sinusitis	3 yr	M	Canada
1663-95	Blood				Canada
3352-95	Urine		85 yr	M	Canada
4556-96	Eye		50 yr	F	N.Y.
4557-96	Eye		83 yr	F	N.Y.
1145-97	Blood	Sepsis	66 yr	F	S.C.
3492-97	Blood		63 yr	M	Canada
4628-97	Blood		78 yr	M	Ga.
37-98	Blood			M	Ga.
2949-98	Nasopharyngeal				Ariz.
4294-98	Blood	Sepsis	2 mo	M	S.C.
4420-98	Blood		11 yr	M	Tenn.
4545-98	Nasopharyngeal				Ariz.
4709-98	Eye		2 mo	F	Ga.
5083-98	Blood	Sepsis	2 mo	M	Mo.
4154-99	Gastric		79 yr	F	Canada
4199-99	Blood		1.8 yr	F	Ga.
4791-99	Nasopharyngeal				Ariz.
4792-99	Nasopharyngeal				Ariz.

1.4.3 Production of anti-*Dolosigranulum pigrum* in rabbits

After its identification as a novel species, further investigation of the organism was performed by producing antibody to it using four rabbits (R3-R6). The rabbit antisera (R3,R4, R5 and R6) employed in this study were the gift of Professor G.W.A. Dick. The antisera were prepared by Professor Dick at the animal unit of the University of Surrey. Immunizations and all related procedures were carried out under the relevant Home Office regulations (circa

1987). The resulting *D. pigrum* antisera were initially employed in a study of the antigenic relationship of *D. pigrum* to biochemically similar species and the results were published by Aguirre and co-workers (Aguirre et al., 1993, pp. 608-612). The methods employed appear in Appendix E.

These four rabbit antisera formed the basis of further work into the possible identity of bacterial antigen(s) detected in early acute MS CNS tissue. All rabbit antisera to *D. pigrum* were screened for antibody to Brain Heart Infusion medium (BHI) using BHI on 12% polyacrylamide gels followed by Western blotting, as described in 2.7 and no antibodies were detected. Similarly, human control and MS control sera had no detectable antibody to BHI using these techniques (D. Gay, personal communication, January 16, 2014).

1.4.4 Experiments using rabbit antisera

Investigations using the rabbit antisera R3, R4, R5 and R6 were begun. Cryostat sections of the frozen spinal cord tissue from which *D. pigrum* was isolated were treated with R3 anti-*D. pigrum* serum at a titre of 1/100, and stained using biotinylated goat anti-rabbit, streptavidin-horse radish peroxidase and colour development with 3,3′-diaminobenzadine tetrahydrochloride (DAB). Positive reactions with an antigen ('R3.89') were found throughout these tissues (Figure 1.9), with the strongest and most frequent being within activated macrophages in the meninges, in the perivascular (Virchow-Robin) spaces and in the microglia. The pre-immune serum showed no reaction, and no reaction was found when a wide range of neurological tissue from normal control brain tissues and a variety of other neurological diseases were tested. However, similar positive reactions were found in other MS tissues, but all were from early stages of the disease (D.

Gay, personal communication, December 22, 2008). All of the four rabbit antisera were subsequently tested and it was seen that R3 reacted most strongly, producing a signal at 1/100 dilution. R4 produced a positive reaction in the tissues at 1/10 dilution, R6 in 1/5 dilution and R5 at less than a 1/5 dilution.

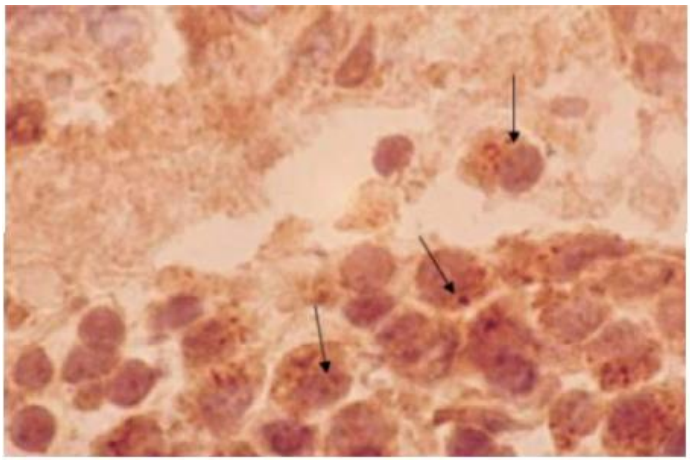

Figure 1.9. Cryostat section of spinal cord which grew *Dolosigranulum pigrum* showing R3.89 antigen (arrowed) in cytoplasmic vesicles. Treated with rabbit 3 anti-D. pigrum serum at 1/100, and stained using biotinylated goat anti-rabbit and streptavidin-horse radish peroxidase. Colour development 3,3′-diaminobenzadine tetrahydrochloride (DAB). Positive reactions (antigen R3.89) were found throughout these tissues, the strongest and most frequent within activated macrophages within the meninges, in the perivascular spaces, and in microglia (Figure provided by Dr. F. W. Gay).

This immuno-histological observation using rabbit antisera led to a postulation that the antigen(s) in MS tissue were in fact small transportable (extracellular) bacterial proteins, possibly produced by *D. pigrum*, that might be a causative factor in the development of MS plaques. The idea of identifying the tissue antigen(s) by relative reactivity was the subject of an undergraduate thesis by C.W. Gay (2007). In these investigations the small transportable protein antigens of *D. pigrum* were investigated, using SDS-PAGE and Western blotting techniques (Figure 1.10). The investigations used the four rabbit antisera (R3, R4, R5, R6) to *D. pigrum* to detect *D. pigrum* peptides and the possible identity

and pathogenicity of these peptides was discussed. The study defined up to ten distinct small peptides (denatured) that all mirrored the characteristic tissue reactivity to the different *D . pigrum* rabbit antisera.

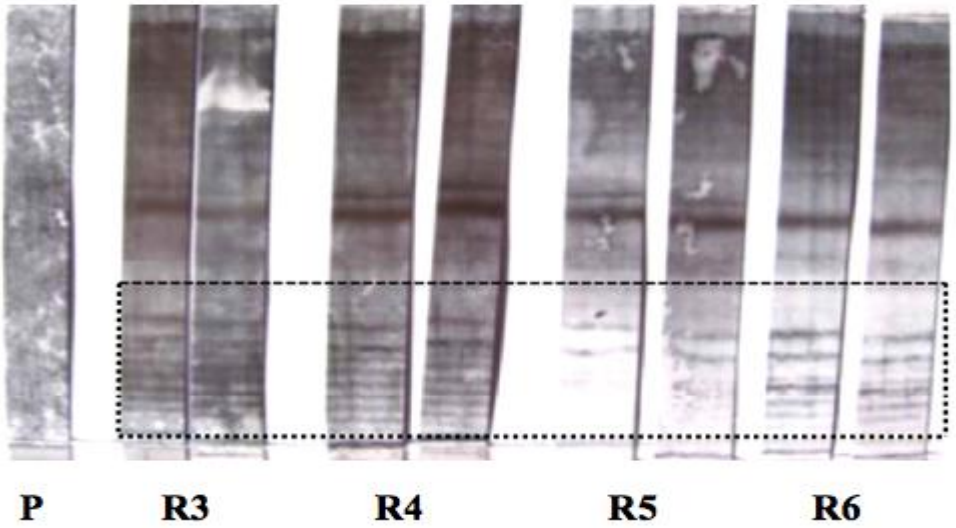

Figure 1.10. Western blot of an extracellular (no cells) preparation of *Dolosigranulum pigrum* reacted against rabbit 3, 4, 5 and 6 anti-*Dolosigranulum pigrum* antibody, with a pre-immune rabbit negative control (P) from R3. All the other rabbit pre-immune sera (R4-6) also showed a lack of bands. This Western blot illustrates the comparative band strength produced by the four rabbit antisera. It also illustrates the range of low molecular weight bands. (C. Gay, 2007).

1.4.5 Epidemiology of *Dolosigranulum pigrum*

The ability of *D. pigrum* to survive in inhospitable conditions was demonstrated by 16S rRNA detection of it among *Staphylococcus* species, *Moraxella* species and other bacteria, in clean rooms where spacecraft are assembled at three separate centres in America (Moissl et al., 2007, pp. 509-521).

The pathogenic potential and worldwide occurrence of *D. pigrum* has recently been highlighted in several reports (Table 1.2). In China it has been implicated in a case of acute cholecystitis accompanied by acute pancreatitis (Lin et al., 2006, pp. 2298-2299) and has been found in a case of synovitis in the USA (Hall et al., 2001, pp. 1202-1203) and septic arthritis in Norway (Johnsen, Rønning, Onken, Figved & Jenum, 2011, pp. 85-87), whilst in the Netherlands it has been isolated from a case of ventilator associated pneumonia (Hoedemaekers, Schulin, Tonk, Melchers & Sturm, 2006, pp. 3461-3462), and in France it was found in a case of non-ventilator associated pneumonia and septicaemia (Lécuyer et al., 2007, pp. 3474-3475). In these studies, and in a review article by LaClaire and Facklam (2000a, pp. 2001-2003), the organism was found to be susceptible to most commonly used antibiotics, with the exception of erythromycin. Reduced susceptibility to chloramphenicol and trimethoprim-sulfamethoxazole was detected in some strains. *D. pigrum* was also discovered in the sputum of cystic fibrosis patients by Bittar and co-workers (Bittar et al., 2008) using PCR and cloning of the the purified PCR products (Table 1.2). Their finding of eight clones of the bacterium amongst a total of 392 clones of 55 other bacterial species illustrate the complexity and diversity of the flora found in the disease. The authors concluded that in conjunction with other bacteria, *D. pigrum* could be a cause of pulmonary infections in cystic fibrosis. These findings were corroborated by a later study (Armougom et al., 2009, pp. 1151-1154).

Table 1.2. Reported clinical isolates of *Dolosigranulum pigrum* since LaClaire & Facklam's (2000) study showing diagnosis and geography.

Date	*Source*	*Clinical diagnosis*	*Locale*
2001	Blood culture	Synovitis/sepsis	USA
2006	Blood culture	Cholecystitis/pancreatitis	China
2006	Bronchial secretions	Pnemonia	Netherlands
2007	Broncho-alveolar lavage	Pneumonia	France
2008	Sputum	Cystic fibrosis	France
2009	Sputum	Cystic fibrosis	Germany
2011	Synovial biopsy	Septic arthritis	Norway

1.4.6 Association of *Dolosigranulum pigrum* with multiple sclerosis

As already stated, antibodies to *D. pigrum* low molecular weight antigens have been found to react with an immune complex in very early pre-demyelinating lesions in MS tissues (Figure 1.7). This research project aimed to look at the potential relationship of *D. pigrum* with MS by measuring antibodies to *D. pigrum* in MS cases and in matched controls. If leakage of bacterial products into the CNS occurs *via* the posterior sinuses in MS then it was important to confirm that the upper respiratory tract is the natural habitat of *D. pigrum*. When this study commenced in 2006 there was speculation that *D. pigrum* was part of the normal flora of the nasal cavity and the naso-pharynx (LaClaire & Facklam, 2000a, pp. 2001-2003). Recently evidence has been found that

suggests this is the case (Cho, Jung, Kim & Chang, 2011, pp. 18-22) and may play a protective role in colonization resistance against potential upper respiratory tract pathogens such as *Streptococcus pneumoniae* and *Haemophilus influenzae* (Laufer et al, 2011, pp. 1-6). Additionally, Bogaert et al. (2011, para. 1- 8) found evidence of seasonal variation in the microbial flora of children with an increase in bacterial numbers and diversity of both potential pathogens and normal flora, including *D. pigrum* in the autumn and winter. These findings correlate with seasonal increases in respiratory infections,(White et al., 2009, p. 196; Kinlan et al., 2009, pp. 588-595), sinusitis and MS (Gay, 1991).

1.5 Aims and objectives of the study

Aims

The bacterial toxins hypothesis in MS postulates that bacterial transportable toxins generated by the flora of the human nasopharynx access the central nervous system and are implicated in the pathogenesis of the disease.
The bacterium *D. pigrum* was originally isolated from and first identified in acute multiple sclerosis tissues. The aims of this study were:

1) Determine if the organism is a normal inhabitant of the nasopharyngeal flora in man (Figure 2.1).
2) To explore the relationship between *D. pigrum* infection and MS (Figure 2.2).

Objectives

- To design and use an enzyme linked immunosorbant assay to screen for antibody (IgG) to *D. pigrum* in the serum of MS patients and a control group.

- To characterize, using Western blotting, any antibodies found in the enzyme linked immunosorbant assay and establish any points of similarity between them by comparison of their electrophoretic banding pattern.
- To screen for *D. pigrum* in nasal and other specimens using bacteriological cultural techniques.
- To develop and use primers to *D. pigrum* and set up a polymerase chain reaction test to screen for the presence of the bacteria in nasal and other specimens sent for microbiological culture.
- To use the results from this project to test the bacterial toxins hypothesis of MS and assess the feasibility of using the ELISA and Western blotting tests used for this project to develop specific diagnostic tests for MS, enabling early diagnosis and treatment of the disease before serious damage occurs to the brain and spinal cord.

Chapter 2:
Methods

2.1 Flow charts showing methodology for the two aims of the project

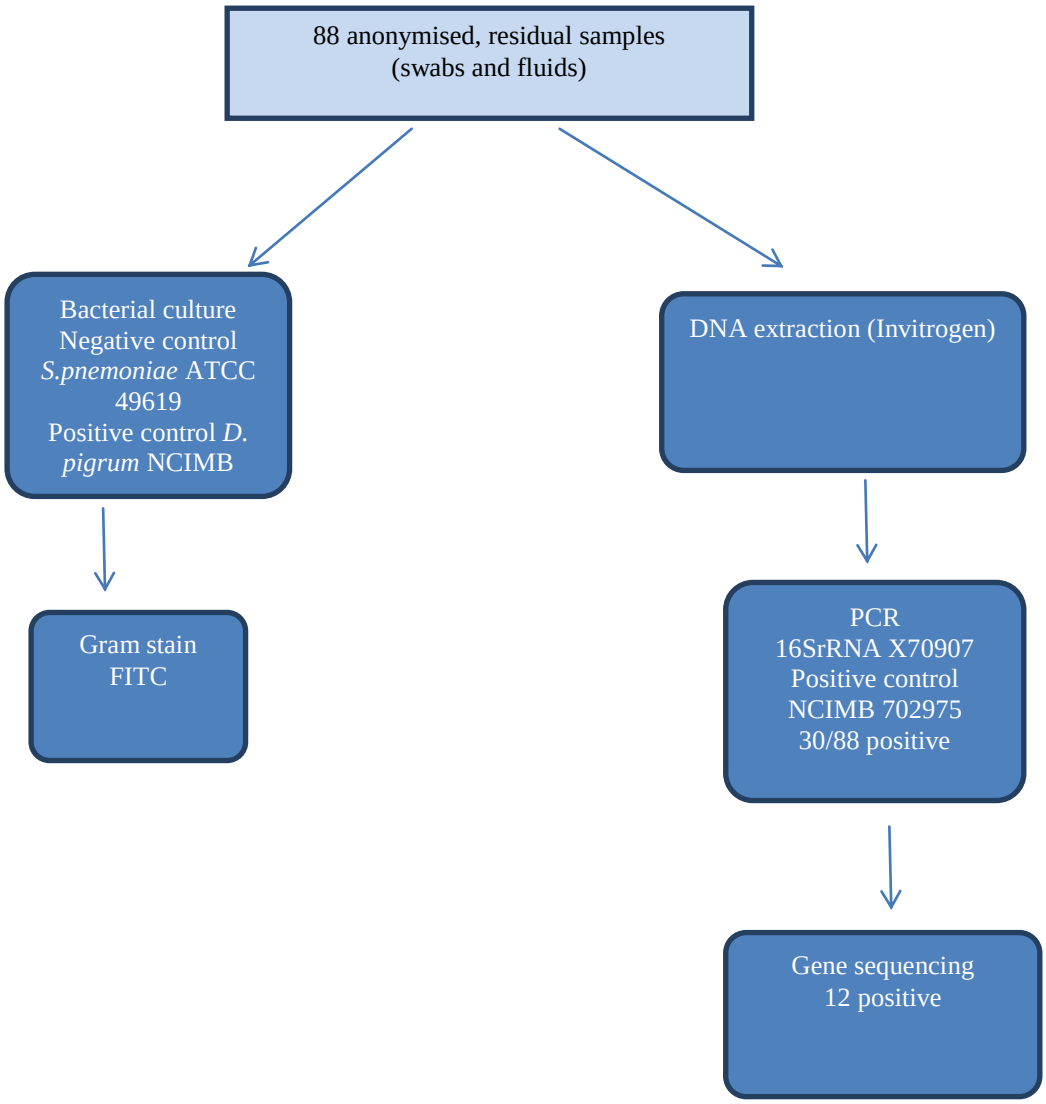

Figure 2.1. Project aim 1.
To determine if the organism is a normal inhabitant of the nasopharyngeal flora in man using eighty eight anonymised residual swab samples and various body fluids from Colchester microbiology

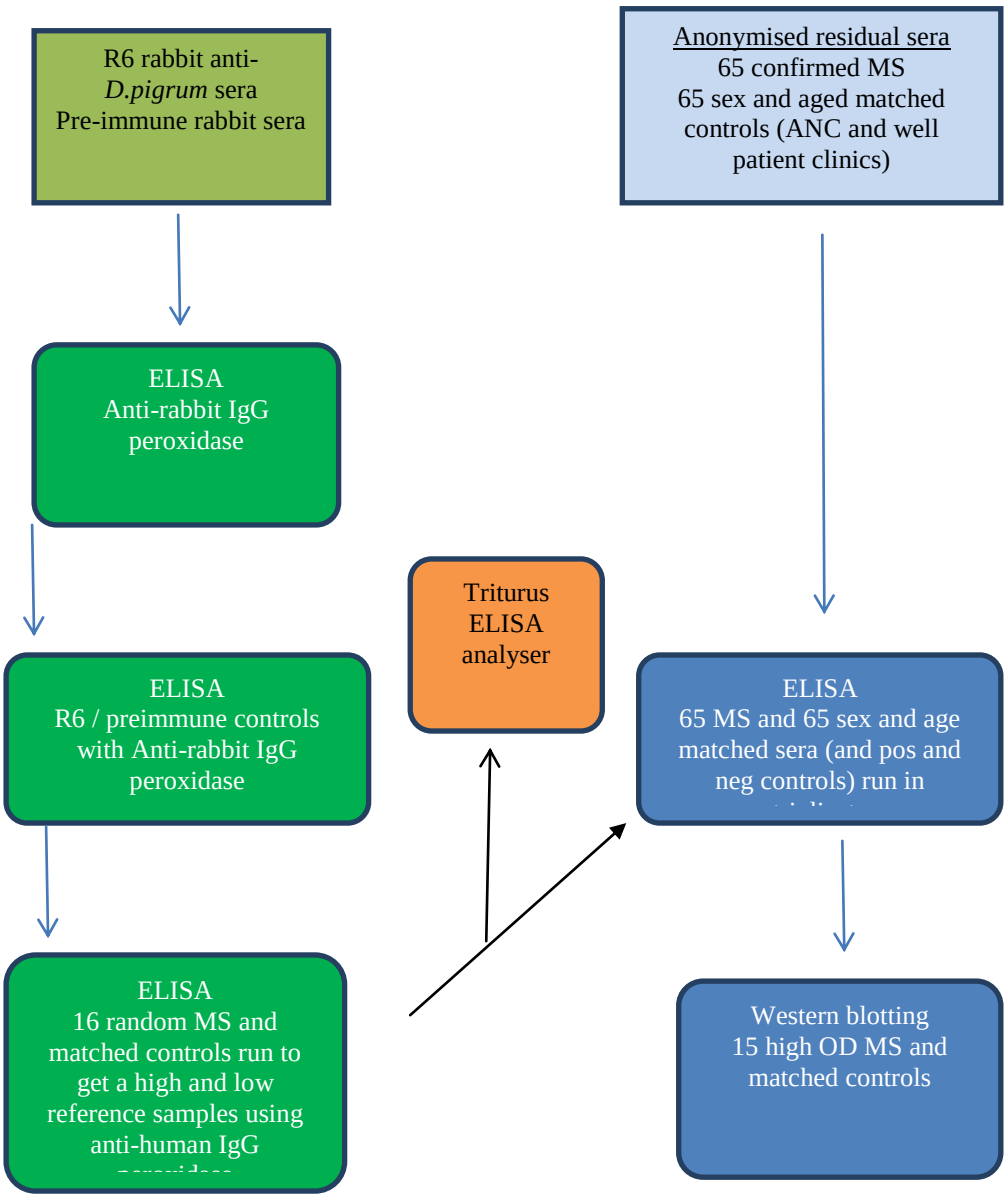

Figure 2.2. Project aim 2.
To explore the relationship between *Dolosigranulum pigrum* infection and multiple sclerosis by measuring antibody to the bacterium in multiple sclerosis patients and in matched controls using sixty five anonymised residual sera from confirmed cases of multiple sclerosis from the Neurology Unit, Queens Square, London and sixty five anonymised residual sera from age and sex matched controls from Colchester microbiology

2.2 Specimens used for the project

2.2.1 Ethical approval

Approval for this project was granted by the National Research Ethics Service (NRES) and the Research and Development Department of the Colchester Hospital University Foundation Trust (Appendix A1).

2.2.2 Specimens used for culture, fluorescence microscopy and polymerase chain reaction

Eighty eight anonymised residual samples (respiratory, eye and ear swabs (in Amies transport media) and various body fluids) sent for diagnostic testing to the Microbiology Department, Colchester Hospital University Foundation Trust, were used for this project. The specimens were collected in September and October 2008 after routine microbiological testing and kept frozen at -20°C, as were cultures of *Dolosigranulum pigrum* NCIMB 702975. These were inoculated into Amies transport media, pending bacterial culturing for *Dolosigranulum pigrum*, fluorescence microscopy, Western blotting (WB) and PCR. *Dolosigranulum pigrum* NCIMB 702975 survival was checked before culture. Microscopy was begun and was found to be satisfactory.

2.2.3 Specimens used for enzyme linked immunosorbent assay and Western blotting

Test group: Sixty five anonymised residual sera from confirmed cases of MS from the Institute of Neurology, National Hospital for Neurology and Neurosurgery, Queen Square, London WC1N 3BG. Specimens were kept frozen at –20°C until required.

Control group: Sixty five anonymised residual sera from routine ante-natal testing, and well-patient testing from the Serology Department, Microbiology Department, Colchester Hospital University Foundation Trust. These were age and sex matched to the test group, and the sera were kept frozen at –20°C.

2.3 Culture for *Dolosigranulum pigrum*

All the specimens were cultured onto Columbia agar (Oxoid CMO331) with 5% horse blood (TCS Biosciences, HBO34) added (BA). The plates were incubated for 7 days in 5% CO_2 at 37°C. Tiny α haemolytic colonies were sub-cultured onto BA purity plates and incubated as before. If Gram staining revealed Gram positive cocci (see Figure 3.4b) then the presence of *D. pigrum* was tested for using R3 (see 1.4.3 Production of anti-*D. pigrum* in rabbits) and FITC-labelled goat anti-rabbit IgG (Sigma, F1262), using *D. pigrum* NCIMB 702975 as a positive control, and *Streptococcus pneumoniae* ATCC 49619 as a negative control. The controls and tests were made by inoculating 2 ml of phosphate buffered saline (PBS) (BBL FTA haemagglutination buffer, phosphate buffered saline pH 7.2 +/- 0.1, Becton Dickinson 211248) with harvested cultures from Columbia blood agar plates, and adjusting the suspension so that the optical densities equated to a McFarland 2 standard. The suspensions were then centrifuged at 120 × *g* for 10 min and the pellet washed once in PBS and re-suspended in 1 ml of PBS before being cultured onto Columbia blood agar plates to check that were no losses to controls or test organisms.

2.4 Fluorescein isothiocyanate method to detect *Dolosigranulum pigrum*

One 10 µl loop of well mixed test or control suspension was then emulsified in 25 µl of PBS on a glass slide and left to air dry before fixing with acetone, which was left until the acetone evaporated.

The rabbit antisera (R3) was stored at -20°C undiluted. Twenty five µl of a 1/50 dilution of R3 in PBS was added to each fixed preparation on a slide and incubated in a damp chamber at room temperature for 30 min. Following incubation, the slides were washed with PBS and blotted dry. Twenty five µl of a 1/50 dilution of FITC-labelled goat anti-rabbit conjugate (Sigma, F1262) in PBS was then added and the slides left in the dark in a damp chamber for 30 min at room temperature. The slides were then washed with PBS and blotted dry. After mounting with Detect IF mountant (Axis-Shield) the slides were examined for fluorescence using an ultra violet microscope with a 450-480 nm excitation 500 nm emission filter.

2.5 DNA extraction method

PureLink Genomic DNA kits from Invitrogen (K1820-01) were used for all extractions (see Appendix B.1).

2.5.1 Preparation of naso-pharyngeal aspirates, mucous and swabs

Naso-pharyngeal aspirates (whole samples) were centrifuged at 9000 × *g* for 9 min and the pellet used for DNA extraction.

Approximately 10 ml of mucous was combined with 180 µl of PureLink Genomic Digestion Buffer and 20 µl of 20 µg ml^{-1} PureLink Proteinase K and incubated at 55°C. The mixture was then shaken occasionally, until the mucous was digested. The sample was then centrifuged at 9000 × *g* for 9 min and the pellet used for DNA extraction.

The tip of the specimen swabs was aseptically cut off and placed directly into the PureLink Genomic Lysis/Binding Buffer /Lysozyme mixture, and the sample was then used for DNA extraction.

2.5.2 DNA extraction

Lysozyme (Sigma, L6876) was added to 300 µl of PureLink Genomic Lysis/Binding Buffer to obtain a concentration of 20 mg ml^{-1}. This mixture was then vortex mixed for 5 s. The specimens (as prepared in 2.4.1) were then added and the mixture incubated at 37°C for 1 h. After incubation, 20 µl of proteinase K and 200 µl of genomic lysis/binding buffer were added and then vortex mixed for 5 s. The sample was then incubated at 55°C for 1 h. If swabs were used these were now removed using aseptic techniques, squeezing them against the side of the tube to ensure that as much material as possible was extracted. Two hundred µl of 100% v/v ethanol was added and the mixture was vortex mixed for 5 s, before all the fluid was removed from the tube and added to a microfuge tube with a PureLink spin column. During the development of the PCR test it was found that even though the amount of ethanol in the final eluted product was miniscule it was interfering with the PCR. Centrifugation of open microfuge tubes after the ethanol had been removed, and leaving the spin columns to sit at room temperature for half an hour after the elution, significantly reduced ethanol

contamination and did not result in DNA contamination (C. Gay and McGenity, personal communication March 2009).

The microfuge tube was centrifuged at 10000 × *g* for 1 min. The tube was then discarded and the spin column transferred to a new microfuge tube. Five hundred µl of PureLink wash buffer 1 was then added and the open tube centrifuged at 10000 × *g* for 1 min. The tube was then discarded and the spin column placed into a new microfuge tube. 500 µl of PureLink wash buffer 2 was added and the open tube centrifuged at 13000 × *g* for 5 min. The spin column was then placed into a fresh microfuge tube which was left open at room temperature in a fume cupboard for 30 min. At the end of this time 25 µl PureLink elution buffer was added and the tube was then left open at room temperature in a fume cupboard for 15 min. The tube was then closed and centrifuged at 13000 × *g* for 1 min. The spin column was then discarded and the DNA extract kept frozen at -20°C.

2.6 Polymerase chain reaction method

2.6.1 Primers

The primers and PCR methods were designed and developed by C.W. Gay and T. McGenity as part of a MSc project (Gay, 2009). Four sets of primers were developed from 16S rRNA (acc. X70907) of *D. pigrum* (Figure 2.1).

Figure 2.3. Location of primers used in the investigation of *Dolosigranulum pigrum* 16S rRNA gene (X70907).
Primer set 'a': Location 166F and 1251R
Primer set 'b': 165F and 1270R
Primer set 'c': 226F and 1243R
Primer set 'd': 447F and 747R
F = Forward, R = Reverse.

Primer set 'a' gave the best results and was therefore used for this project.

Amplicons of this primer were 1085 base pairs (Gay, 2009).

2.6.2 Master mix formulation

Fifty μl of reactions contained 1 μl of each primer made up to 10 pmol ml^{-1} each, 5 μl of 1mM (total) deoxynucleotide triphospates, 1μl of 2.5 U Taq polymerase and 5 μl of the PCR buffer (Qiagen), and 2 μl of the template DNA.

Amplification was performed in Gene Amp PCR system 97000 thermal cyclers (Applied Biosystems) as follows: 94°C for 5 min, 35 cycles of 94°C for 1 min, 58°C for 1 min and 72°C for 2 min, with a final elongation step of 72°C for 7 min and a holding temperature of 4°C. Amplification of the target DNA was confirmed by agarose gel electrophoresis (1% w/v in TAE buffer at 100 V) and ethidium bromide staining.

During the development of the primer it was demonstrated that a slightly higher annealing temperature of 58°C did not affect the sensitivity of the reaction so this was used in the hope of improving specificity.

2.6.3 Electrophoretic separation of polymerase chain reaction products

One % agarose gels in 1% TAE (Appendix B.2) were used for separating PCR products, by electrophoresis. PCR product (5 μl) was combined with loading dye (1 μl) (Appendix B.2) and the mixture added to the gel. Five μl of DNA ladder (Appendix B.3) was added in a separate lane.

A 0.7% agarose gel to visualise DNA was performed on some extracted samples and demonstrated that DNA was being sucessfully extracted.

Development of gels

The gels were treated with ethidium bromide (0.5 μg ml^{-1}) for 20 min and then soaked in tap water for 20 min. They were then examined under UV light (254 nm) (Bio-Rad Chem Doc System).

2.6.4 Purification of products for sequencing

Twelve positive samples (bands formed at 1085 bp, or 1085 bp and 1000bp) were selected for sequencing (Table 3.2). The PCR products were purified using QIA Quick PCR Purification Kit (Qiagen). Three μl of the purified PCR product was then run in a 1% agarose gel at 100 mV for 40 min to check that sufficient PCR product was present. The products were then sent for sequence analysis to Gene Service, 2 Cambridge Science Park, Milton Road, Cambridge CB4 0FE, for sequencing (Table 3.2).

The results from Gene Service were analysed firstly using the internet tool Bioedit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) which is a sequence alignment editor. Sequences were analysed and if they were of interest (showing variable bases) they were copied into Notepad (Microsoft) and then analysed using BLAST (Basic Alignment Tool) (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Results were then sent as FASTA sequence codes to CLUSTALW2 multiple sequence alignment programme http://www.ebi.ac.uk/Tools/clustalw2/index.html).

2.7 Development of an enzyme linked immunosorbent assay method for testing for *Dolosigranulum pigrum* antibody in serum

2.7.1 Preparation of microtitre trays

Preliminary studies by Dr. Bruce Roser's team at Cambridge Biostability, had shown that the production of an ELISA method to look for antibodies to *Dolosigranulum pigrum* were practical (D. Gay, personal communication, March 2009). These studies used a 1/5 dilution of a 5 day culture of *D. pigrum* in brain heart infusion broth and Rabbit 6 anti-*D. pigrum* pre and post immune serum. It was decided to expand upon this to produce an ELISA to screen MS and matched controls from human sera for antibody to *D. pigrum*.

A culture of *D. pigrum* National Collection of Industrial, Food and Marine Bacteria (NCIMB) 702975 was inoculated into 200 ml of brain heart infusion broth and incubated with shaking at 50 rpm at 37°C for 5 days. This was used as a stock solution and was stored at -20°C. The culture was demonstrated to be both viable and pure by sub-culturing onto a blood agar plate and incubating at 37°C for 5 days. The stock solution from the 5 day culture was then centrifuged at 1000 × *g* for 20 min and the supernatant taken off. The supernatant was diluted 1 in 5 with PBS and 200 µl was pipetted into each well of a 96 well microtitre tray (Sterilin 611 F96) and left overnight at 4°C. The wells were then washed five times using a Triturus ELISA analyser and then 350 µl of a 1% solution (in PBS) of a blocking agent, chicken egg albumin (Sigma A5503), was added to each well (reagents and materials, Appendix B4). The tray was left overnight at 4°C and then washed five times in PBS using the Triturus.

2.7.2 Establishing a methodology for an enzyme linked immunosorbent assay test for anti-*Dolosigranulum pigrum*

Previous work had led to the development of *D. pigrum* antibodies in rabbits. All rabbits, R3-R6, were found to be hyper-immune to *D. pigrum* with visual bands on Western blotting. The fluorescent antibody titres of R6 were found to be 1/250,000 and therefore suitable for the ELISA investigations (D. Gay, personal communication, December, 2008) and was used as a positive control. The pre-immune serum from this animal was used as the negative control. Preliminary investigations conducted by Cambridge Biostability (D. Gay, personal communication, December, 2008) had indicated that a 1/100 serum dilution in PBS with a 1/10,000 dilution of anti-rabbit IgG peroxidase conjugate in PBS were the optimum concentrations for establishing an ELISA test for screening for anti-*D. pigrum*. A range of titrations using R6 and R6 pre-immune rabbit sera as positive and negative controls respectively showed that a 1/100 serum dilution and 1/16,000 dilution of anti-rabbit IgG peroxidase conjugate was the ideal concentration (Table 2.1) .

Table 2.1 Titration of R6 and anti-rabbit IgG peroxidase conjugate to find the ideal concentrations.
Dolosigranulum pigrum (NCIMB 702975) supernatant from a 5 day culture was diluted 1 in 5 with PBS and 200 µl was pipetted into each well of the microtitre tray (Sterilin 611 F96) and left overnight at 4°C. The wells were then washed five times and then 350 µl of a 1% solution (in PBS) of a blocking agent, chicken egg albumin (Sigma A5503), was added to each well (reagents and materials, Appendix B4). The tray was left overnight at 4°C and then washed five times in PBS. Dilutions of the anti-rabbit IgG conjugate and R6 were then added along with 1/100 pre-immune R6 sera.

R6 →

	Negative Pre-immune R6 1/100	1/25	1/50	1/75	1/100
1/4000	1.414	2.714	2.907	2.905	2.936
1/8000	0.672	1.748	1.828	1.8	1.818
1/12000	0.401	1.293	1.385	1.413	1.352
1/16000	0.281	0.858	0.97	0.973	0.963

↓ Anti-rabbit IgG peroxidase conjugate

2.7.3 Multiple sclerosis and matched control sera for *Dolosigranulum pigrum* antibody screen

Sera from sixty five confirmed cases of MS were age and sex matched with asymptomatic control sera and then coded by Stephen Ball, the senior Biomedical Scientist in the serology department of Colchester microbiology. All sera, including the positive and negative controls, were then tested 'blind' in triplicate, and the samples were not decoded until after analysis.

2.7.4 Enzyme linked immunosorbent assay method to detect *Dolosigranulum pigrum* antibody in human sera

The method was automated using a Triturus analyzer and sixteen randomly selected MS and control sera were run on a plate lined with uninoculated BHI using the methodology in 2.6.2 and the optical densities were extremely low indicating that antibodies to brain were not present (Table 2.2). Three runs using the same sixteen randomly selected MS and control sera and methodology were performed on microtitres plates lined with a 1/5 dilution of *D. pigrum* in BHI with anti-human IgG peroxidase in conjunction with the positive and negative rabbit controls with anti-rabbit IgG peroxidase (Sigma A6029) to ensure that the method worked with human sera, and to optimize the wash cycle and incubation times (2.6.3). It was shown that a 1/100 dilution of human serum and a 1/16,000 dilution of the anti-human IgG peroxidase conjugate were optimal. Three samples containing PBS instead of sera were also used with a 1/16,000 dilution of the anti-human IgG peroxidase conjugate and gave optical densities of 0.153, 0.240 and 0.176 respectively. The runs were also used to find a sample with a high optical density that could be used as a positive reference sample (serum 36490, see Table 3.4) and a sample with a low optical density that could be used as a negative reference sample (serum 36590, see Table 3.5).

Table 2.2. Detection of antibodies to brain heart infusion in multiple sclerosis and control sera.
200 µl Brain Heart Infusion broth (neat) was pipetted into each well of the microtitre tray (Sterilin 611 F96) and left overnight at 4°C. The wells were then washed five times and then 350 µl of a 1% solution (in PBS) of a blocking agent, chicken egg albumin (Sigma A5503), was added to each well (reagents and materials, Appendix B4). The tray was left overnight at 4°C and then washed five times in PBS before 1/100 MS and control sera were added. 1/16,000 dilution of 100 µl anti-human IgG peroxidase conjugate and tetramethylbenzadine buffer/chromagen added with a stopping solution of sulphuric acid.

MS ID	OD	Control ID	OD
36452	0.052	36475	0.063
36455	0.059	36478	0.071
36461	0.063	36505	0.073
36483	0.057	36507	0.082
36487	0.062	36510	0.082
36490	0.066	36512	0.063
36494	0.071	36541	0.054
36497	0.075	36543	0.058
36498	0.071	36546	0.038
36516	0.065	36570	0.045
36519	0.056	36572	0.045
36526	0.050	36586	0.062
36529	0.067	36590	0.070
36550	0.078	36595	0.070
36555	0.077	36601	0.056
36561	0.075	36604	0.051

Using prepared microtitre trays (see 2.6.1). The sera were diluted 1/100 and 100 µl was added to each microtitre well. The plate was then incubated at 37°C for 1 h, and the wells washed five times using 350 µl per well (30 s soak) with PBS. A 1/16,000 dilution of 100 µl anti-human IgG (whole molecule) peroxidase conjugate (Sigma A6029) was added, and the microtitre plate incubated at 37°C for 30 min (stationary). The plates were then washed five times again, using 350 µl per well (30 s soak) with PBS.

One hundred µl tetramethylbenzadine buffer/chromagen (TMB) (BioRad 492430) was added to each well, and the microtitre plate incubated at room

temperature for 30 min. One hundred µl stopping solution (1 N sulphuric acid) was then added to each well, and the plate read on the Triturus using a read filter of 450 nm and a reference filter of 620 nm.

2.8 Western blotting

Fifteen of the MS sera demonstrating high optical density in the *D. pigrum* ELISA along with their age and sex matched controls were tested using Western Blotting.

2.8.1 Gel preparation

The gel was produced between two glass plates using the clamping system of the Mini-Protean II slab cell (BIO-RAD). A spacer thickness for the casting plates of 0.75 mm was used which employed a total of 4.2 ml of gel monomer. The gel used was a discontinuous (Laemmli) gel consisting of a 4% stacking gel which concentrated the sample to give better band resolution, and a 12% separating gel which facilitates separation of the molecules. The separating gel monomers were prepared by adding all the ingredients except ammonium persulphate and tetramethylethylenediamine (TEMED). These were de-aerated using a vacuum pump for 15 min. The ammonium persulphate and TEMED were then added and mixed. A comb was placed into the gel sandwich. A mark of 1 cm was drawn from the top of the casting plate and the separating gel was delivered up to this mark using a pipette, taking care not to mix the solution with air. The gel was then overlayed carefully with distilled water and left to polymerize for 1 h.

The ingredients for the stacking gel were then combined except ammonium persulphate and TEMED (reagents and materials Appendix B5). The gel was de-aerated using a vacuum pump for 15 min. The ammonium persulphate and TEMED were then mixed in. The water above the separating gel was taken off and dried with blotting paper before the stacking gel was poured. This was then left to polymerize for 1 h. The gel sandwiches and clamp assembly were then removed from the casting stand and attached to the cooling core. A 1/5 dilution of the stock electrode buffer was then made with distilled water to 300 ml. The inner cooling core was lowered into the lower buffer chamber and 115 ml of buffer was added to the upper buffer chamber so that it reached a level halfway between the two glass plates of the gel sandwich. The remainder of the electrode buffer was then poured into the lower buffer chamber so that at least the bottom 1 cm of the gel was covered. Any air bubbles formed at the bottom of the gel were carefully removed to ensure good electrical contact.

2.8.2 Electrophoresis and Western blotting

These studies used the supernatant of the 5 day culture of *D. pigrum* (NCIMB 702975) in brain heart infusion (as used in the ELISA part of the project). A 1/3 dilution of the *D. pigrum* supernatant in sample buffer (reagents and materials, Appendix B6) was heated to 98°C for 4 min. After cooling, 400 µl was added to the stacking gel under the electrode buffer. The cooling clamp was then removed and the lid of the top of the buffer chamber was closed to fully enclose the cell. The electrical leads were connected and the electrophoresis was run at 140 V for 1 h. After electrophoresis, the gel was removed and placed in equilibration buffer (Tris 3.03 g, glycine 14.4 g, de-ionised water, methanol v/v 200 ml, pH

8.3 ued at 4 °C) for 15 min to remove buffer salts and detergents. The nitrocellulose membrane was cut to the size of the gel and orientation of the membrane was identified by nicking the top left edge. Using forceps, the membrane was slowly added at an angle of 45° to the transfer buffer and allowed to soak for 15 min. The pre-cut filter paper and fibre pads were soaked in transfer buffer, and the buffer tank half filled with transfer buffer . (Tris 3.03 g, glycine 14.4 g, de-ionised water, methanol v/v 200 ml, pH 8.3 ued at 4 °C). The cooling unit was installed in the buffer chamber next to the electrodes. The gel holder cassette was assembled, and the gel and membrane inserted according to the manafacturer's instructions. The Western blot was then run at 100 V for 1 h. After this time the membrane was washed with PBS and blocked with 2% milk/PBS for 30 min. The membrane was then cut into strips and stored at 4°C in 0.2% milk/saline for 12 h.

For immunoprobing each strip was then placed in a sterile universal bottle and 0.5 ml of MS or matched control sera was added. The bottles were then placed on a roller and mixed for 1 h. Two runs were performed using the following sera.

Table 2.3. Multiple sclerosis and matched control sera used for Western blotting using the BioRad Mini-Protean.

Run1	
MS sera	***Matched control sera***
036563	036603
036528	036587
036454	036597
036455	036601
036456	036475
036460	036541

Run 2	
MS sera	***Matched control sera***
36548	36574
36524	36660
36529	36506
36521	36572
36464	36584
36484	36570
36494	36576
36488	36505
36496	36478

After rolling the sera were discarded and the strips washed in 20 ml of 0.2% milk/PBS three times. 0.5 ml of the second antibody goat anti-human IgG at 1/1000 dilution was added and the strip rolled for 1 h. The strip was washed three times with 0.2% milk/PBS and then 0.5 ml of a 1/1000 dilution of anti-goat IgG labelled with horse radish peroxidase (Appendix B5) was added and rolled for 1 h. The strips were then washed three times with distilled water and then reacted with diaminobenzidine (DAB) colour reagent (Sigma D6815) for 30 min. The strips were then washed three times with distilled water, air dried and mounted.

Chapter 3:
Results

3.1 Samples tested by culture, fluorescein isothiocyanate and polymerase chain reaction for *Dolosigranulum pigrum*

Eighty eight samples were tested as shown in Table 3.1. They ranged from newborn to 78 years old, and 47 (53.4%) were male and 41 (46.6%) were female.

3.1.1 Fluorescein isothiocyanate and culture results for *Dolosigranulum pigrum*

To test for the presence of *D. pigrum* by fluorescein isothiocyanate (FITC) (Figure 3.4a), 88 swabs and fluids sent for diagnostic testing to the Microbiology Department, Colchester Hospital University Foundation Trust, were cultured for *D. pigrum* on blood agar plates along with *D. pigrum* NCIMB 702975 (Figures 3.1, 3.2, 3.3, 3.4b) which was used as a positive control and *S. pneumoniae* ATCC 49619 which was used as a negative control. Microbiological results for pathogen screening prior to this study were also recorded (Table 3.1). In this study there were 22 samples that grew colonies that resembling *D. pigrum* NCIMB 702975 which were Gram positive cocci (19 throat swabs, 1 nose swab, 1 quinsy pus and 1 naso-pharyngeal aspirate (NPA) but all tested FITC- negative for the organism. Twenty one were from mixed cultures and the FITC test was repeated taking a sweep of the mixed growth which also proved negative for *D. pigrum*. Finally, all 88 samples were tested by directly inoculating the samples onto the slide and testing with FITC. Again, all were negative for *D. pigrum* using the FITC test.

Figure 3.1. *Dolosigranulum pigrum* NCIMB 702975 on blood agar after 72 h incubation at 37°C in 5% CO_2. Tiny non-haemolytic colonies of less than 0.5 mm in diameter.

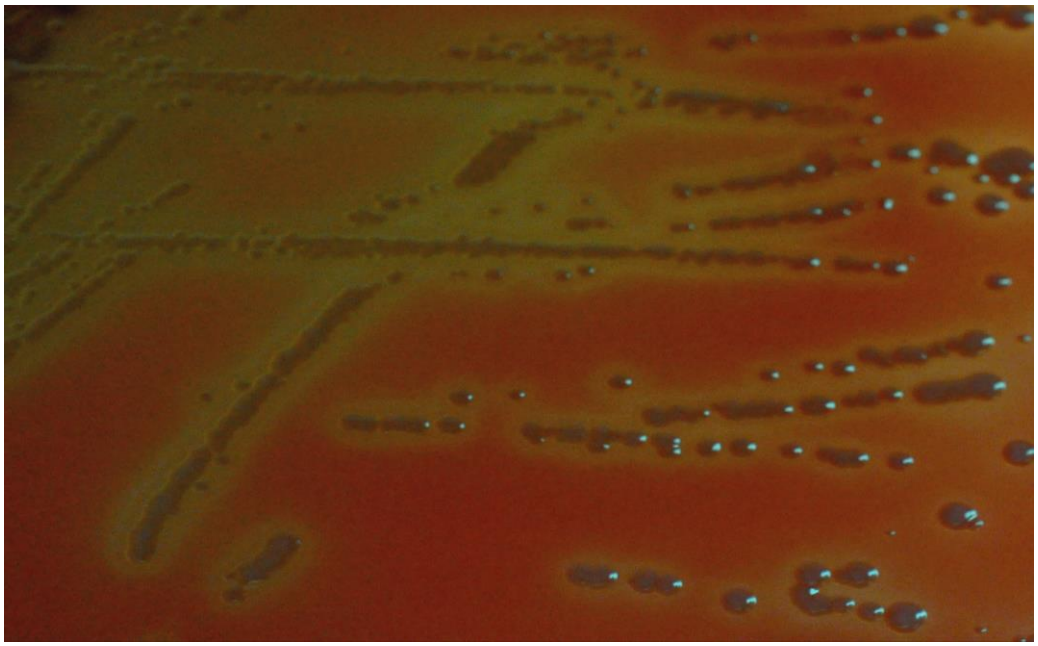

Figure 3.2 *Dolosigranulum pigrum* NCIMB 702975 on blood agar after 5 days incubation at 37°C in 5% CO_2. Backlit plate to demonstrate α haemolytic colonies of 1 mm in diameter.

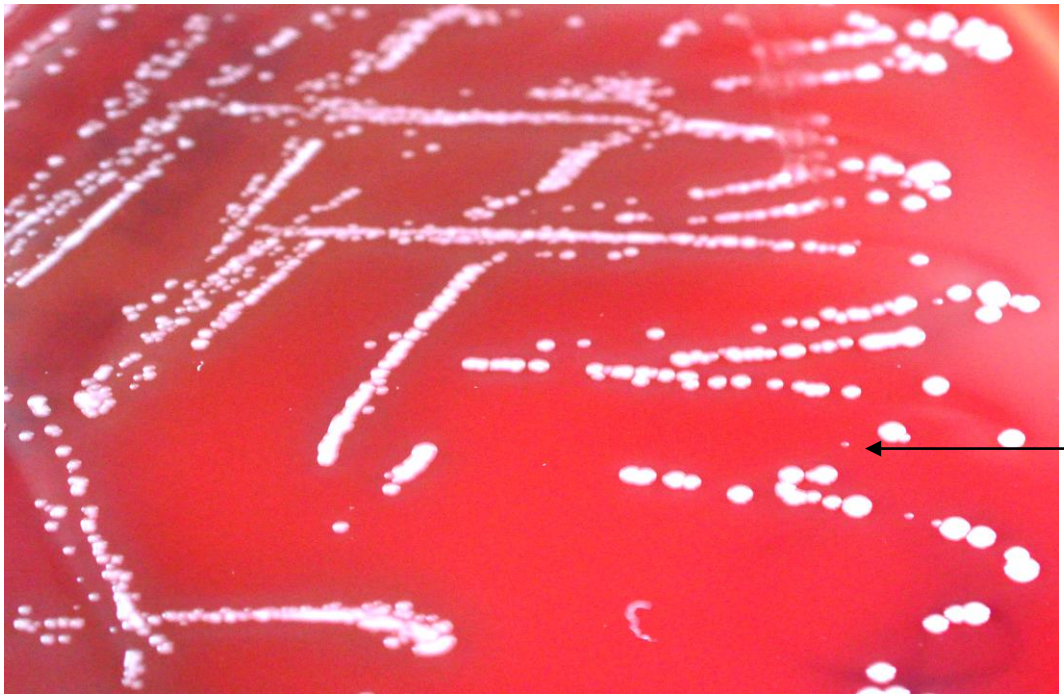

Figure 3.3. *Dolosigranulum pigrum* NCIMB 702975 on blood agar after 5 days incubation at 37°C in 5% CO_2. Top lit plate showing white colonies of 1 mm in diameter. Tiny colonies of less than 1mm (arrowed) were routinely seen on culture but reverted to normal colonies when subcultured.

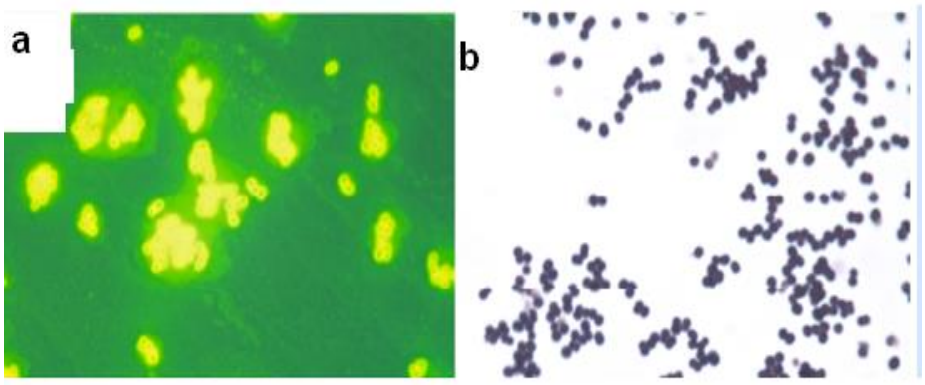

Figure 3.4. Microscopy from *Dolosigranulum pigrum* NCIMB 702975 on blood agar after 5 days incubation at 37 °C in CO_2.
(a). *Dolosigranulum pigrum* NCIMB 702975 stained with anti-rabbit FITC showing fluorescence, using rabbit anti-*Dolosigranulum pigrum* antibody (R3).
(b). Gram stain of *Dolosigranulum pigrum* NCIMB 702975 demonstrating the characteristic short chains and clusters of 1– 1.5 µm Gram positive cocci.

3.1.2 Polymerase chain reaction results for *Dolosigranulum pigrum*

Of the 88 samples tested (Table 3.1, Figures 3.5-3.11) 30 samples demonstrated bands at 1085 base pairs (34%) ranging from 8 days old to 74 years old, with 17 of the positives being male (56.7%) and 13 being female (43.3%). Analysis showed the highest percentage of *D. pigrum* PCR positivity with infants (Figure 3.12).

Analysis of the sequencing results showed that of the twelve PCR products sent (Gene Service, 2 Cambridge Science Park, Milton Road, Cambridge CB4 0RE), three produced good signals but did not match the *D. pigrum* sequence, and two produced mixed signals that indicated there were mixed amplicons. The other seven were all good matches for *D. pigrum* (Table 3.2, Table 3.3).

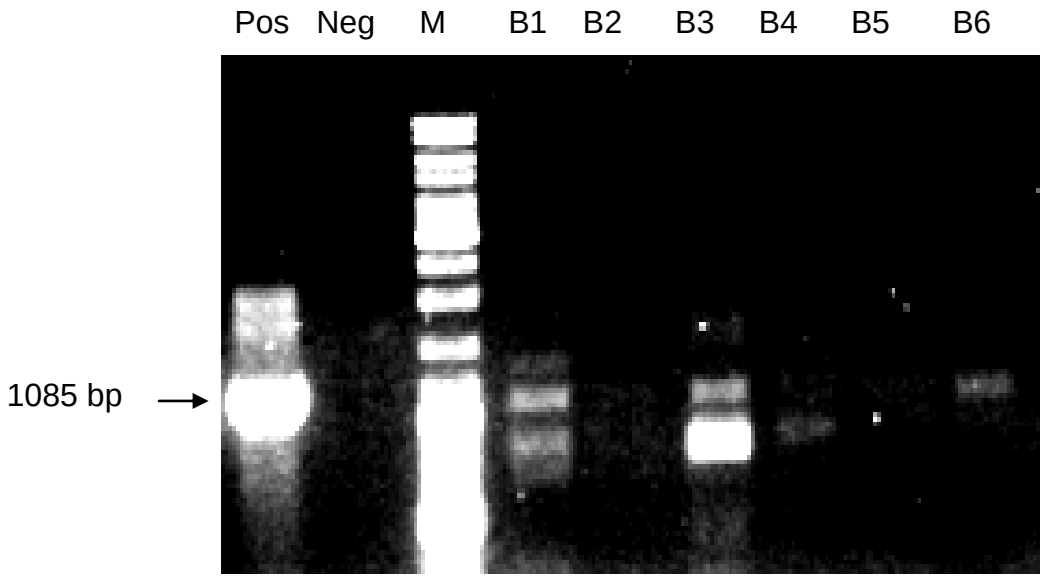

Figure 3.5. PCR run samples B1 – B6
Agarose gel (1%) showing positive result for amplicon at 1085 base pairs with *Dolosigranulum pigrum* positive control and B6, positive results at 1085 bp with a second band at 1000 bp for B1 and B3, and a negative result for B2, B5 and the negative control. M is the marker lane with a DNA ladder.
Gel run at 100 V, for 35 min. Stained with ethidium bromide.

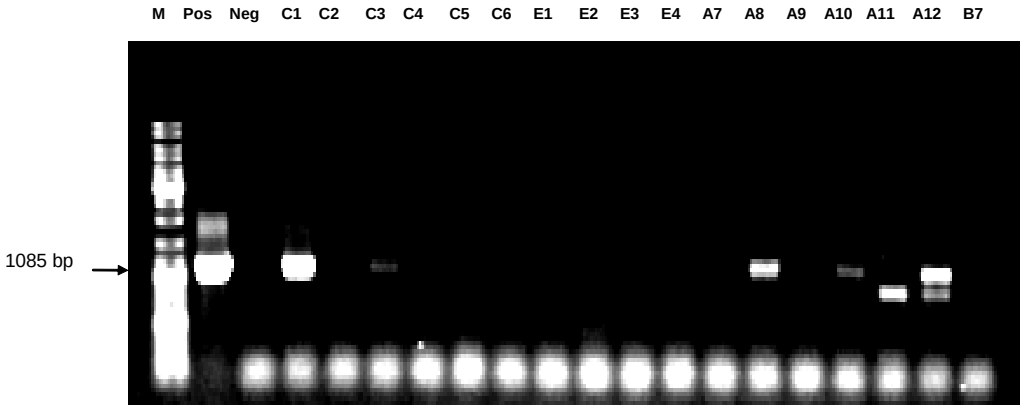

Figure 3.6. PCR run 141108A
Agarose gel (1%) showing positive result for amplicon at 1085 base pairs with *Dolosigranulum pigrum* positive control (Pos) and samples C1, C3, A8 and A10 positive result for amplicon at 1085 base pairs with a second band at 1000 bp with sample A12, and a negative results for all other samples and the negative control (Neg). M is the marker lane with a DNA ladder.
Gel run at 100 V, for 35 minutes. Stained with ethidium bromide.

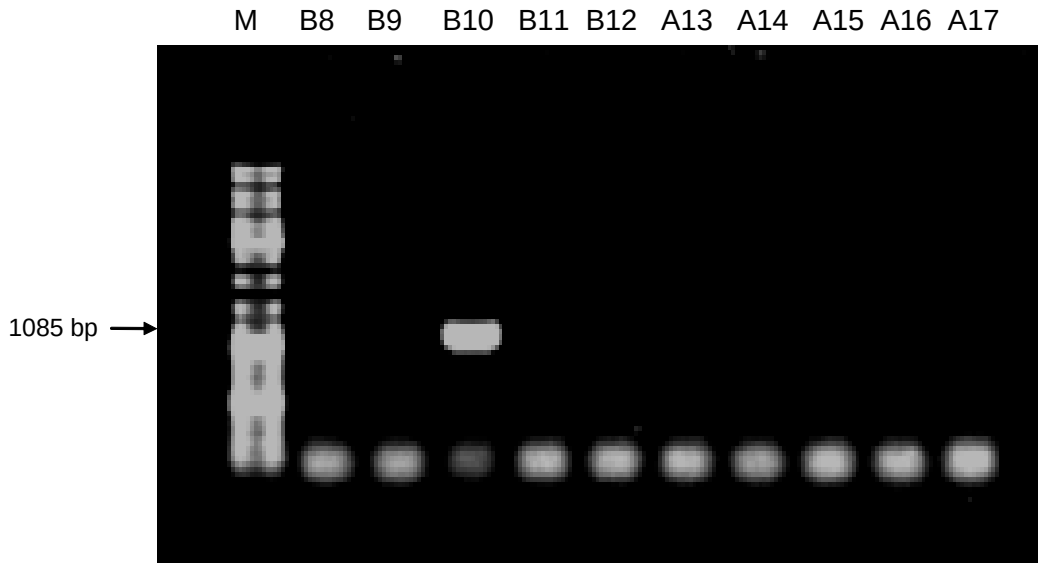

Figure 3.7. PCR run 141108B
Agarose gel (1%) showing positive result for amplicon at 1085 base pairs with sample B10, and a negative results for all other samples. M is the marker lane with a DNA ladder.
Gel run at 100 V for 35 minutes.Stained with ethidium bromide.

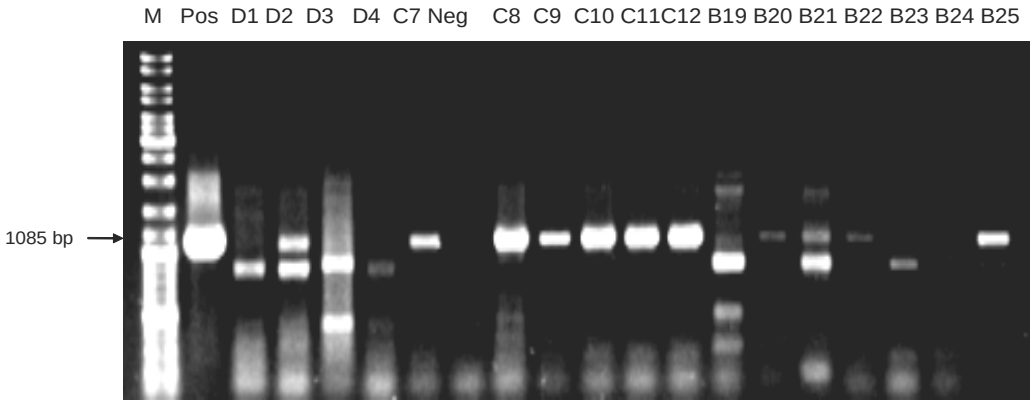

Figure 3.8. PCR Run 181108A
Agarose gel (1%) showing positive result for amplicon at 1085 base pairs with *Dolosigranulum pigrum* positive control (Pos) and samples C7-C12, B20, B22, B25, with a second band at 1000 bp with D2 and B21, and a negative result for all other samples and the negative control (Neg). M is the marker lane with a DNA ladder.
Gel run at 100 V for 45 minutes. Stained with ethidium bromide.

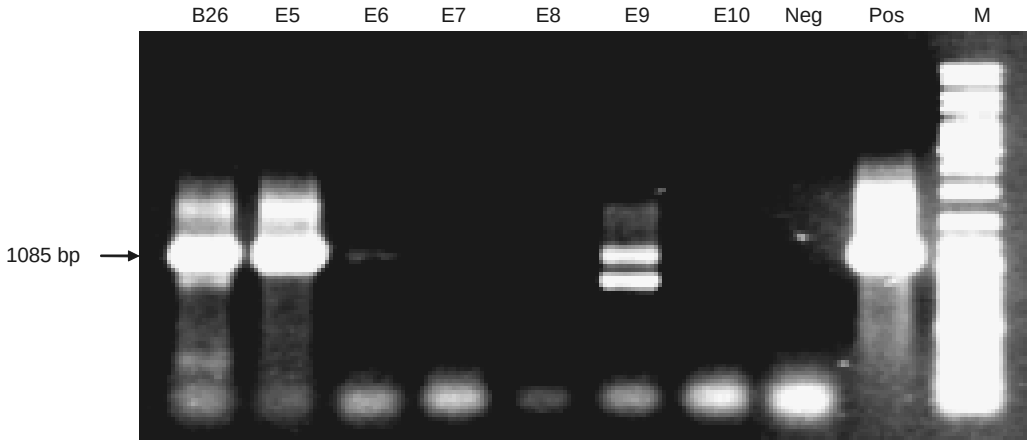

Figure 3.9. PCR Run 181108B
Agarose gel (1%) showing positive result for amplicon at 1085 base pairs with *Dolosigranulum pigrum* positive control (Pos) and samples B26,E5 and E6 , with a second band at 1000 bp with E9, and a negative result for all other samples and the negative control (Neg). M is the marker lane with a DNA ladder.
Gel run at 100 V for 45 minutes. Stained with ethidium bromide.

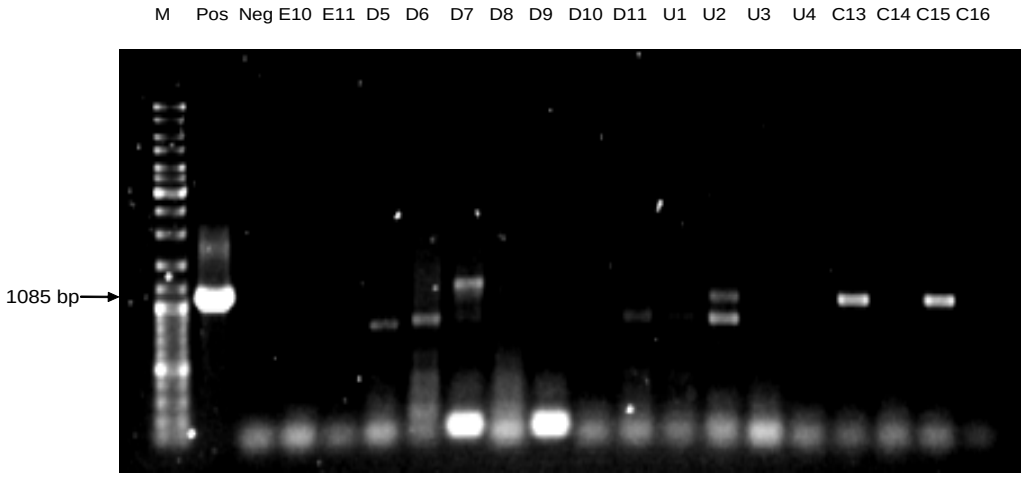

Figure 3.10. PCR run 201108A
Agarose gel (1%) showing positive result for amplicon at 1085 base pairs with *Dolosigranulum pigrum* positive control (Pos) and samples C13, and C15, with a second band at 1000 bp with U2, and a negative result for all other samples and the negative control (Neg).
Gel run at 100 V for 1 h. Stained with ethidium bromide.

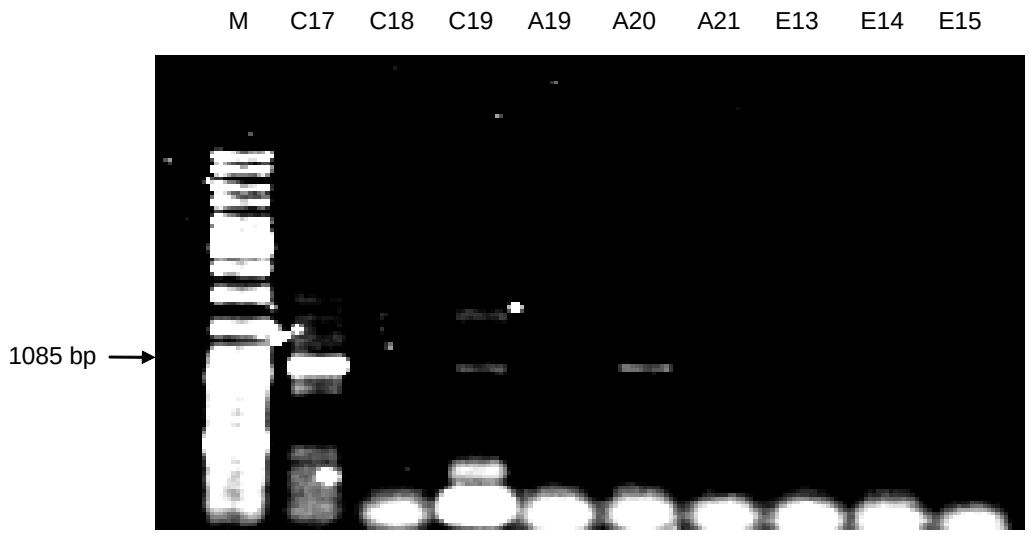

Figure 3.11. PCR run 201108B
Agarose gel (1%) showing positive result for amplicon at 1085 base pairs with sample A20, with a second band at over 1085 bp with C19 and with a second band at 1000 bp with C17, and a negative result for all other samples.
Gel run at 100 V, 400 mA for 1 h. Stained with ethidium bromide.

Table 3.1. Comparison of polymerase chain reaction results with nasal specimen culture results, showing patient demographics and type of specimen.

I.D.	Specimen type	Sex	Age	PCR result *for D. pigrum*	Position of band	Microbiology results prior to project
A1	NS	F	23 y	Neg		SAU +/-
A2	NS	M	15 y	Neg		SAU ++
A3	NS	F	53 y	Neg		NG
A4	NS	F	65 y	Neg		NF
A5	NS	F	18 y	Neg		NG
A6	NS	F	64 y	Neg		SAU +/-
A13	NS	M	8 y	Neg		SPN++
froA14	NS	M	38 y	Neg		SAU+++
						COL+++
A15	NS	M	9 y	Neg		NF
A16	NS	M	40 y	Neg		+SAU
A17	NS	F	28 y	Neg		+/-SAU
A19	NS	F	28 y	Neg		NF
A20	NS	M	71 y	+	1085 bp	MIXCOL+
A21	NS	M	38 y	Neg		SAU+/-
						NF+/-
B7	NS	F	66 y	Neg		MRS+
B8	NS	F	53 y	Neg		NF
B9	NS	F	59 y	Neg		SAU+/-
B10	NS	M	73 y	+	1085 bp	COL+
						NF+
B11	NS	M	9 y	Neg		HSA+
						SAU++
E14	NS	F	78 y	Neg		NF
D1	NMU	F	57 y	Neg		NF
D2	NMU	M	62 y	+	1085 bp	MOR+
					1000 bp	NF++
D4	NMU	M	2 m	Neg		COL++
D5	NMU	F	68 y	Neg		NF
D6	NMU	M	64 y	Neg		NF
D9	NMU	M	22 y	Neg		HSA++
D10	NMU	M	2 y	Neg		NF

Key to Table 3.1. ID = Specimen number, y = years old, m = months old, M = Male, F = Female, NS = Nose swab, NMU = Nasal mucous. Culture results from routine microbiology investigations prior to this study: SAU = *Staphylococcus aureus*, SPN = *Streptococcus pneumoniae*, COL = coliform, MIXCOL = Mixed coliform, MRS = Methicilllin resistant *Staphylococcus aureus*, HAS = *Streptococcus pyogenes*, MOR = *Moraxella* species, NF = Normal flora, NG = No growth.

Table 3.1 continued. Comparison of polymerase chain reaction results with throat specimen culture results, showing patient demographics and type of specimen.

I.D.	Specimen type	Sex	Age	PCR result for *D. pigrum*	Position of band	Microbiology results prior to project
A7	TS	F	19 y	Neg		NF
A8	TS	F	19 y	+	1085 bp	HSA+ NF+
A9	TS	F	24 y	Neg		NF
A10	TS	M	2 y	+	1085 bp	NF
A11	TS	F	2 y	Neg		NF
A12	TS	F	19 y	+	1085 bp 1000 bp	NF
B1	TS	F	3 y	+	1085 bp 1000 bp	NF
B2	TS	M	2 y	Neg		NF
B3	TS	F	37 y	+	1085 bp 1000 bp	NF
B4	TS	F	41 y	Neg		NF
B5	TS	M	10 y	Neg		NF
B6	TS	M	12 y	+	1085 bp	HSA++ NF++
B19	TS	M	20 y	Neg		NF
B20	TS	F	21 y	+	1085 bp	NF
B21	TS	M	17 y	+	1085 bp 1000 bp	NF
B22	TS	F	7 m	+	1085 bp	NF
B23	TS	F	71 y	Neg		CAN++ NF++
B24	TS	M	22 y	Neg		NF
B25	TS	M	1 y	+	1085 bp	NF
B26	TS	M	9 m	+	1085 bp	NF
D8	TMU	F	22 y	Neg		HSG+

Key to Table 3.1. ID = Specimen number, y = years old, m = months old, M = Male, F = Female, TS = Throat swab, TMU = Throat mucous. Culture results from routine microbiology investigations prior to this study: HAS = *Streptococcus pyogenes*, HSG = Haemolytic streptococcus group G, Can = Candida species, NF = Normal flora, NG = No growth.

Table 3.1 continued. Comparison of PCR results with Naso-pharyngeal aspirate (NPA) results, showing patient demographics.

I.D.	Specimen type	Sex	Age	PCR result for *D. pigrum*	Position of band	Microbiology results prior to project
C1	NPA	M	8 m	+	1085 bp	RSV MOR +
C2	NPA	M	7 m	Neg		NF
C3	NPA	M	9 m	+	1085 bp	SPN
C4	NPA	M	3 m	Neg		COL+/-
C5	NPA	M	2 w	Neg		NF
C6	NPA	F	1 m	Neg		NF
C7	NPA	M	8 d	+	1085 bp	HIN++ SPN++ MOR++ NF++
C8	NPA	M	8 m	+	1085 bp	NF
C9	NPA	F	1 m	+	1085 bp	NF
C10	NPA	M	7 m	+	1085 bp	NF
C11	NPA	M	2 m	+	1085 bp	NG
C12	NPA	F	1 y	+	1085 bp	NF
C13	NPA	F	2 m	+	1085 bp	RSV
C14	NPA	M	2 m	Neg		RSV
C15	NPA	M	3 m	+	1085 bp	NF
C16	NPA	M	1 m	Neg		MOR+
C17	NPA	M	2 w	+	1085 bp 1000 bp	NF
C18	NPA	M	3 m	Neg		NG
C19	NPA	M	10 m	+	1085 bp ^1085 bp	SAU+ NF+++

Key to Table 3.1. ID = Specimen number, y = years old, m = months old, d = days old, w = Weeks old, M = Male, F = Female, NPA = Naso pharyngeal aspirate. Results from routine microbiology investigations prior to this study: SAU = *Staphylococcus aureus*, SPN = *Streptococcus pneumoniae*, COL = coliform, MOR = *Moraxella* species, RSV = Respiratory Syncytial Virus, NF = Normal flora, NG = No growth.

Table 3.1 continued. Comparison of PCR results with miscellaneous specimen culture results, showing patient demographics and type of specimen.

I.D.	Specimen type	Sex	Age	PCR result for *D. pigrum*	Position of band	Microbiology results prior to project
B12	ETH	F	66 y	Neg		SAU+
D3	QAS	M	22 y	Neg		SMI+
D7	QAS	F	34 y	Neg		HSA+/- NSA++
D11	AWO	M	50 y	Neg		SPN++
E1	EYE	M	41 y	Neg		NG
E3	EAR	M	NB	Neg		COL++
E4	EYE	M	8 d	Neg		HIN+
E5	EYE	F	74 y	+	1085 bp	SF
E6	EYE	F	40 y	+	1085 bp	SF
E7	EYE	F	29 y	Neg		NG
E8	EAR	F	1 d	Neg		NG
E9	MOU	F	60 y	+	1085 bp 1000 bp	NF
E10	EYE	M	5 d	Neg		NF
E11	ECA	F	58 y	Neg		SAU++
E13	EAR	F	NB	Neg		NG
E15	EAR	M	NB	Neg		NG
E2	QSW	F	24 y	Neg		NF
U1	URI	F	61 y	Neg		ECO++
U2	URI	F	46 y	+	1085 bp 1000 bp	MIXECO++
U3	URI	F	60 y	Neg		ECO++
U4	URI	M	65 y	Neg		NG

Key to Table 3.1. ID = Specimen number, y = years old, d = days old, NB = new born, M = Male, F = Female, EYE = eye swab, EAR = ear swab, MOU = mouth swab, ECA = ethmoid cavity swab, QSW = quinsy swab, QAS = quinsy aspirate, AWO = antral washout, URI = urine. Culture results from routine microbiology investigations prior to this study: SAU = *Staphylococcus aureus*, SPN = *Streptococcus pneumoniae*, COL = coliform, MIXCOL = Mixed coliform, MRS = Methicilllin resistant *Staphylococcus aureus*, HAS = *Streptococcus pyogenes*, HIN = *Haemophilus influenzae*, SMI = *Streptococcus millerii*, HAS = *Streptococcus pyogenes*, NSA = non-sporing anaerobes, ECO = *Escherichia coli*, SF = skin flora, NF = Normal flora, NG = No growth.

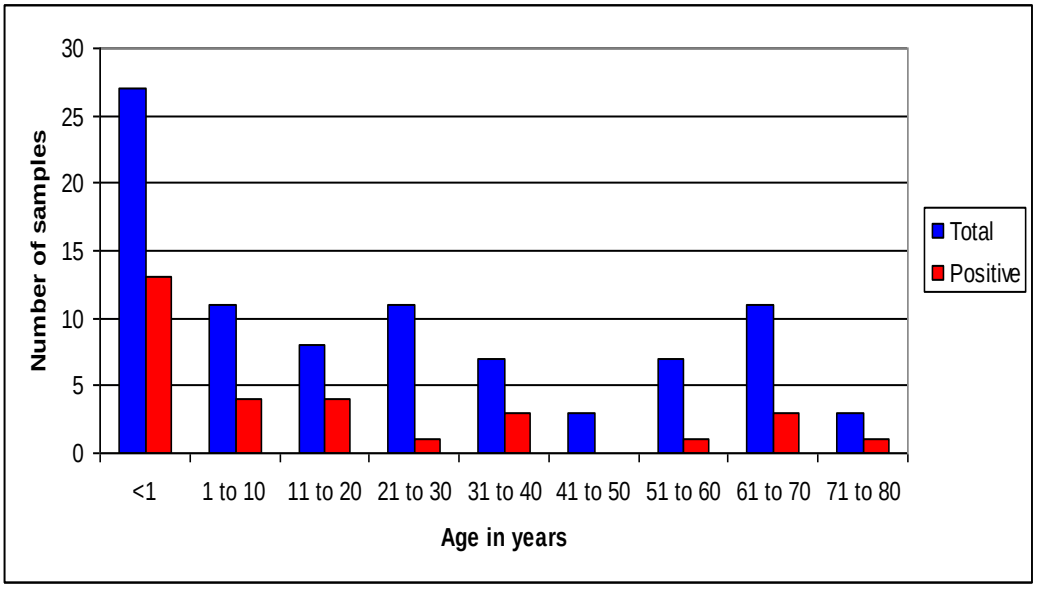

Figure 3.12. Age distribution histogram comparing samples with PCR positive results for *Dolosigranulum pigrum* amplicon at 1085 base pairs with total number of samples for each age range.

Table 3.2 Summary of DNA sequence results

I.D.	*Culture*	*Source*	*Descriptive summary of DNA sequence results*
A20	MIX COL+	N/S	Match to *D. pigrum* sequence
B10	COLI+ NF+	N/S	Match to *D. pigrum* sequence
C7	HIN++ SPN++ MOR++ NF++	NPA	Match to *D. pigrum* sequence
C9	NF	NPA	Match to *D. pigrum* sequence
C10	NF	NPA	Match to *D. pigrum* sequence
C13	RSV	NPA	Match to *D. pigrum* sequence
C15	NF	NPA	Match to *D. pigrum* sequence
A10	NF	T/S	Good sequence, but does not match *D. pigrum* – 100% 16S rRNA sequence identity with *Granulicatella elegans* and *Abiotrophia* species
A12	NF	T/S	Good sequence, but does not match *D. pigrum* – 99% 16S rRNA sequence identity with *Granulicatella elegans* and *Abiotrophia* species
B25	NF	T/S	Good sequence, but does not match *D. pigrum* – 99% 16S rRNA sequence identity with *Granulicatella elegans* and *Abiotrophia* species
A8	HAS NF	T/S	Mixed signals, could mean multiple amplicons
C17	NF	NPA	Mixed signals, could mean multiple amplicons

Key to Table 3.2:

NPA = Nasopharyngeal aspirate
N/S = Nose swab
T/S = Throat swab
I.D. = Sample number

NF =Normal flora
HIN =*H. influenzae*
SPN = *S. pneumoniae*
MOR = Moraxella species
COLI = Coliform
RSV = Respiratory Syncytial virus
HAS = *S. pyogenes*

Table 3.3 Sequence identification BLAST data produced from sequences A10, A12 and B25.

Schedule I.D. and Organism I.D.	***Similarity to sequence data with 100% coverage***
A10 Throat	
Granulicatella elegans AB252689	100% over 340bp
Abiotrophia species AM420130	100% over 340bp
A12 Throat	
Granulicatella elegans AB252689	99% over 360bp
Abiotrophia species AM420130	99% over 360bp
Lactobacillus bacterium EU112069	99% over 360bp
B25 Throat	
Granulicatella elegans AB252689	99% over 325bp
Abiotrophia species AM420130	99% over 325bp

3.2 *Dolosigranulum pigrum* antibody enzyme linked immunosorbent assay results

The results of the ELISA are shown in Tables 3.4 and 3.5. A box and whisker plot of the results indicated that there could be a difference between the MS and the control sera (Figure 3.13). In order to establish if there was a significant difference between the level of anti-*D. pigrum* in the MS and control group the optical densities (OD) were analysed statistically. A preliminary test for the equality of variances indicated that the variances of the two groups ELISA OD were not significantly different $F = 1.028$, $p = 0.455$. Therefore, a two- sample t-test was performed that does not assume equal variances. The mean score of optical densities of the MS group were ($M = 0.911$, $SD = 0.186$, $n = 64$) compared to the control group ($M = 0.804$, $SD = 0.184$, $n = 65$). Using the two-sample t-test for equal variances, $t(9) = 3.306$, $p <= 0.001$ levels of anti-*D. pigrum* in the MS group were significantly different than found in the control group.

Table 3.4 Anti-*Dolosigranulum pigrum* optical density enzyme linked immunosorbent assay results of multiple sclerosis sera

ID	OD1	OD2	OD3	Mean OD
36451	0.722	0.639	0.672	0.678
36452	0.917	0.717	0.777	0.804
36453	0.709	0.552	0.715	0.659
36454	1.332	0.971	1.215	1.173
36455	1.085	0.868	1.092	1.015
36456	1.303	1.177	1.248	1.243
36457	0.528	0.432	0.497	0.486
36458	0.905	0.708	0.842	0.818
36459	0.943	0.922	0.862	0.909
36460	1.242	0.997	1.040	1.093
36461	0.702	0.675	0.770	0.716
36462	1.157	0.822	1.034	1.004
36463	1.062	0.856	0.981	0.966
36464	1.059	0.998	1.096	1.051
36465	0.952	0.832	0.913	0.899
36466	0.873	0.805	0.813	0.830
36483	0.891	0.528	0.836	0.752
36484	1.129	1.021	1.119	1.090
36485	1.045	0.935	1.008	0.996
36486	1.130	1.032	1.072	1.078
36487	1.006	0.871	1.027	0.968
36488	0.995	0.922	1.025	0.981
36489	0.992	0.862	0.961	0.938
36490	1.125	1.096	1.142	1.121
36491	0.543	0.465	0.481	0.496
36492	0.767	0.660	0.714	0.714
36493	0.908	0.756	0.801	0.822
36494	1.269	1.176	1.159	1.201
36495	0.865	0.788	0.889	0.847
36496	1.148	1.146	1.173	1.156
36497	0.815	0.658	0.737	0.737
36498	1.000	0.901	0.983	0.961
36515	0.885	0.756	0.791	0.811
36516	1.034	0.843	0.991	0.956
36517	1.125	0.948	1.034	1.036

ID	OD1	OD2	OD3	Mean OD
36518	1.176	0.917	1.117	1.070
36519	0.893	0.794	0.832	0.840
36520	0.847	0.742	0.811	0.800
36521	1.043	0.961	1.075	1.026
36522	0.862	0.715	0.812	0.797
36523	0.469	0.459	0.453	0.460
36524	1.190	1.058	1.109	1.119
36525	0.820	0.794	0.866	0.827
36526	0.914	0.753	0.885	0.850
36527	1.006	0.821	0.976	0.934
36528	1.174	0.951	1.096	1.074
36529	1.151	1.053	1.175	1.126
36530	0.910	0.708	0.857	0.825
36531	1.062	0.983	1.012	1.019
36548	1.239	1.169	1.187	1.198
36549	INSF	INSF	INSF	INSF
36550	1.086	1.014	1.105	1.068
36551	0.692	0.641	0.756	0.696
36552	0.742	0.621	0.666	0.676
36553	0.759	0.701	0.680	0.713
36554	0.931	0.740	0.938	0.870
36555	0.982	0.927	1.059	0.989
36556	1.077	0.997	1.098	1.057
36557	0.741	0.728	0.729	0.733
36558	1.178	1.149	1.200	1.176
36559	0.806	0.666	INSF	0.736
36560	0.777	0.680	INSF	0.729
36561	1.067	0.881	0.952	0.967
36562	0.793	0.692	0.693	0.726
36563	1.104	0.977	1.182	1.221
Pos	1.439	1.178	1.381	1.333
Neg	0.539	0.481	0.583	0.534

Key to Table 3.4 ID = Sample number, OD1 = 1st optical density reading, OD2 = 2nd optical density reading, OD3 = 3rd optical density reading, Mean OD = mean of optical density readings, INSF = insufficient sample for investigation, Pos = high reference sample, serum 36490, Neg = low reference sample, serum 36590.

Table 3.5 Anti-*Dolosigranulum pigrum* optical density enzyme linked immunoassay results of control sera (age and sex matched with multiple sclerosis sera)

ID	OD1	OD2	OD3	Mean OD
36468	0.760	0.823	1.187	0.923
36473	0.690	0.807	1.088	0.862
36475	0.360	0.371	0.488	0.406
36478	0.693	0.831	1.094	0.873
36499	0.926	1.068	1.480	1.158
36500	0.459	0.462	0.669	0.530
36501	0.562	0.640	0.903	0.702
36502	0.692	0.789	1.113	0.865
36503	0.672	0.745	0.985	0.801
36504	0.439	0.531	0.684	0.551
36505	0.510	0.556	0.800	0.622
36506	0.683	0.747	1.137	0.856
36507	0.350	0.390	0.546	0.429
36509	0.911	1.017	1.425	1.118
36510	0.740	0.828	1.125	0.898
36512	0.703	0.793	1.082	0.859
36514	0.538	0.601	0.879	0.673
36532	0.774	0.864	1.210	0.949
36533	0.661	0.769	1.067	0.832
36534	0.793	0.935	1.340	1.023
36539	0.558	0.602	0.909	0.690
36540	0.534	0.530	0.835	0.633
36541	0.563	0.687	0.986	0.745
36542	0.829	0.921	1.318	1.023
36543	0.703	0.799	1.158	0.887
36544	0.884	0.974	1.426	1.095
36545	0.563	0.609	0.921	0.698
36546	0.413	0.458	0.692	0.521
36564	0.752	0.837	1.266	0.952
36565	0.621	0.687	1.022	0.777
36568	0.734	0.865	1.229	0.943
36569	0.790	0.940	1.363	1.031
36570	0.854	0.933	1.352	1.046
36571	0.590	0.664	0.933	0.729
36572	0.748	0.766	1.172	0.895
36573	0.486	0.535	0.847	0.623
36574	0.705	0.808	1.078	0.864
36575	0.709	0.822	1.184	0.905
36576	0.670	0.688	0.997	0.785
36577	0.611	0.671	0.969	0.750
36578	0.468	0.517	0.753	0.579
36579	0.623	0.667	0.965	0.752
36580	0.760	0.842	1.220	0.941
36581	0.585	0.642	0.888	0.705
36582	0.831	0.941	1.310	1.027
36583	0.464	0.486	0.718	0.556
36584	0.848	0.938	1.313	1.033
36585	0.621	0.729	0.994	0.781
36586	0.723	0.770	1.158	0.884
36587	0.622	0.713	1.015	0.783
36588	0.641	0.716	1.032	0.796
36590	0.380	0.416	0.630	0.475
36591	0.689	0.856	1.203	0.916
36592	0.608	0.725	1.036	0.790
36593	0.514	0.580	0.809	0.634
36594	0.802	0.961	1.369	1.044
36595	0.350	0.389	0.483	0.407
36596	0.677	0.751	1.111	0.846
36597	0.702	0.839	1.205	0.915
36599	0.785	0.872	1.220	0.959
36600	0.630	0.733	1.015	0.793
36601	0.815	0.900	1.358	1.024
36602	0.386	0.454	0.703	0.514
36603	0.504	0.564	0.841	0.636
36604	0.749	0.828	1.209	0.929
Pos	0.872	1.056	1.406	1.111
Neg	0.416	0.417	0.740	0.524

Key to Table 3.5 ID = Sample number, OD1 = 1st optical density reading, OD2 = 2nd optical density reading, OD3 = 3rd optical density reading, Mean OD = mean of optical density readings, INSF = insufficient sample for investigation, Pos = high reference sample, serum 36490, Neg = low reference sample, serum 36590.

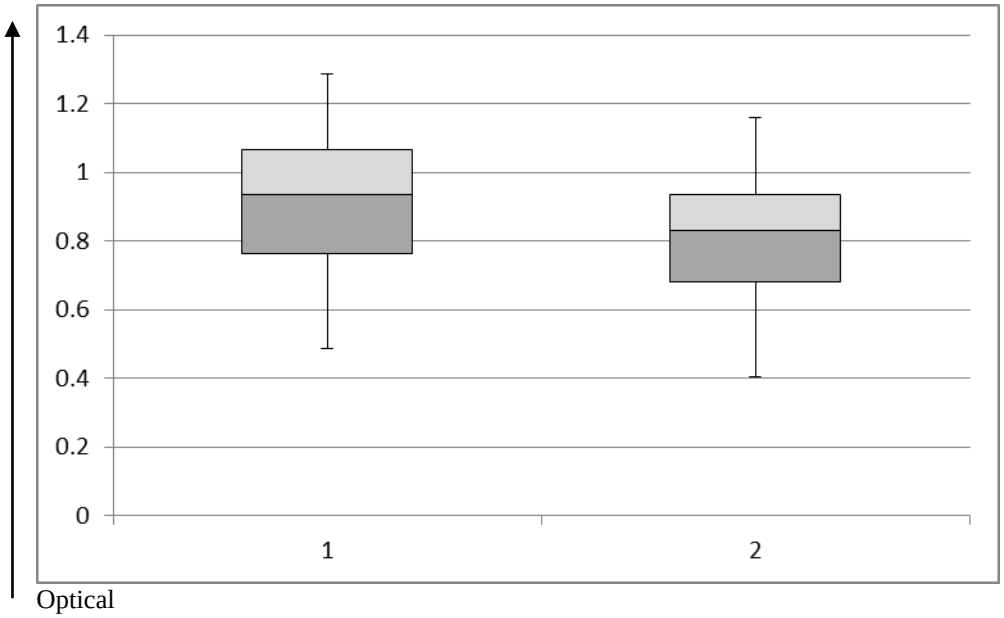

Figure 3.13. Box and whisker plot of anti-*Dolosigranulum pigrum* enzyme linked immunoassay optical densities results showing median, 1st and 3rd quartiles and maximum and minimum values.
1 – Multiple sclerosis sera
2 - Control sera (age and sex matched with multiple sclerosis sera)

3.3 Western blotting of *Dolosigranulum pigrum* against multiple sclerosis and matched control sera using R6 sera

Examination of the blots (Figure 3.14) showed no common bands with R6 and any sera, even the MS sera that had demonstrated elevated levels of anti-*D. pigrum* when tested by ELISA. This was in contrast to the bands found in a previous study using rabbit *D. pigrum* antisera R6 (C.W. Gay, 2007).

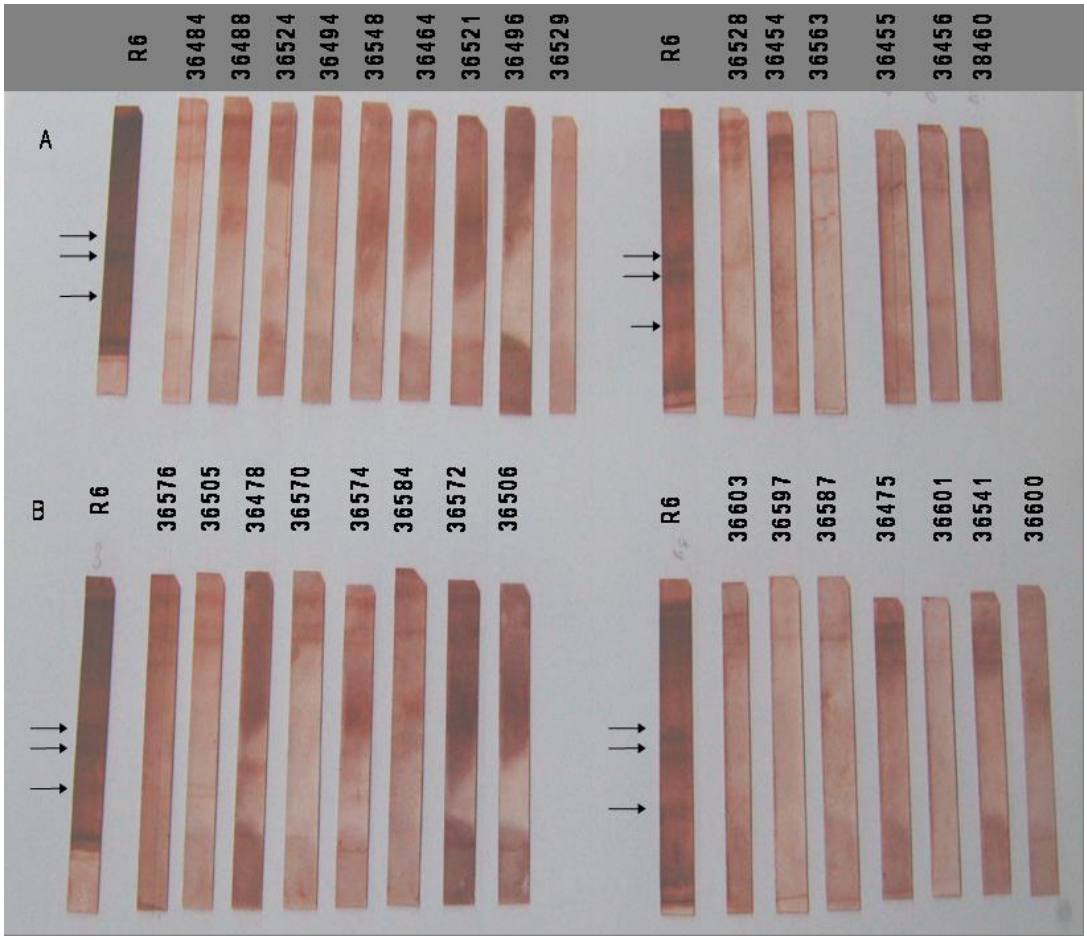

Figure 3.14 . Western blotting of *Dolosigranulum pigrum* culture supernatant against row A multiple sclerosis sera and row B age and sex matched sera using the Mini-Protean II (Bio-Rad) and 0.45 µm nitrocellulose membranes.
The strips were immunoprobed using goat anti-human IgG (Sigma) followed by anti-goat IgG horseradish peroxidase (Sigma) and diaminobenzadine (Sigma). No common bands were seen with R6 and the patients sera. R6 are Western blots from a previous study using the same equipment and *Dolosigranulum pigrum* culture supernatant but with polyclonal rabbit antisera immunoprobed with goat anti-rabbit IgG and then anti-goat IgG horseradish peroxidase with bands demonstrating antibody to *Dolosigranulum pigrum* arrowed (C.W.Gay, 2007).

Chapter 4:
Discussion

4.1 Comparison of culture methods and fluorescein isothiocyanate techniques with polymerase chain reaction for the study of *Dolosigranulum pigrum* in multiple sclerosis

FITC labelled antibodies have been successfully employed to detect a variety of bacteria (Hoff, 1988, pp. 2949-2952). The fact that this technique did not prove successful in this study, failing to find *D. pigrum* in any of the samples, is probably due to a lack of sensitivity because of the absence of suitable selective media and the polyclonal nature of the rabbit antibody used (C.W. Gay, 2007) leading to the inability to differentiate *D. pigrum* from the huge number of phenotypically similar bacteria found in respiratory samples (Laclaire & Facklam, 2000a, pp.2001-2003; Ruoff, 2002, pp. 1129-1133). This difficulty can be seen in the fact that the majority of reports of clinical isolates of *D. pigrum* have come from sites that possess little or no other bacteria and the organism has thus been present in pure culture (Tables 1.1 and 1.2). A search for a selective medium for *D. pigrum* to aid in the isolation of the organism is unlikely to be successful as the bacteria are sensitive to penicillin and vancomycin (Cho et al., 2011, pp. 18-22; Hall et al., 2001, pp. 1202-1203; Hoedemaekers et al., 2006, pp. 3461-3462; Lécuyer et al., 2007, pp. 3474-3475) as are many of the other catalase negative, Gram positive cocci found in the nasopharynx (Laclaire & Facklam, 2000a, pp.2001-2003; Ruoff, 2002, pp. 1129-1133). Yet the intuitive problems inherent in isolating *D. pigrum* from mixed cultures are not the only difficulty encountered when trying to culture these slow growing bacteria. Two cultures of the naso-pharyngeal samples, C11 and C18, in this study yielded no

growth even after prolonged incubation, despite the PCR detecting *D. pigrum*. This suggests that either the bacteria were non-viable or so fastidious in their requirements that they were unable to grow on the bacteriological plates used. The difficulties of culturing this bacterium have also been encountered by Lucuyer (Lécuyer et al., 2007, pp. 3474-3475) in a patient with pneumonia and septicaemia. *D. pigrum* was cultured in abundance from bronchial aspirates but failed to grow from the blood. *D. pigrum* was identified from both sites using 16S rRNA gene sequencing. It would seem that the culture of *D. pigrum* using conventional bacteriological techniques is still fraught with difficulties. There are also problems with the phenotypic identification of this slowly growing bacterium. Phenotypically *D. pigrum* closely resembles bacteria such as *Ignavigranum* species, *Alloiococcus* species and *Facklamia languida* (Ruoff, 2002, pp. 1129-1133). It is described as being Gram positive and appearing in pairs and tetrads, being catalase and oxidase negative with disc tests for pyrrolidonyl arylamidase (PYR) and leucine aminopeptidase (LAP) showing a positive reaction (Laclaire & Facklam, 2000a, pp.2001-2001; Ruoff, 2002, pp. 1129-1133) but this is not enough to differentiate it from a number of phenotypically similar organisms (Table 4.1). Looking at Table 4.1 it would seem that the use of production of PYR and LAP for identification is a relatively simple means of identification but this is complicated by strain variability to these reactions, with *Lactococcus* strains in particular showing variable reactions in the PYR test (Ruoff, 2002, pp. 1129-1133). Similarly the use of microscopic morphology to differentiate *Dolosigranulum pigrum, Facklamia languida, Rothia mucilaginosa, Gemella haemolysans* from *Vagococcus, Lactococcus, Ignavigranum, Abiotrophia, Granulicatella, Gemella* spp. other than *G.*

haemolysins or *Facklamia* other than *Facklamia languida* is in practice a highly subjective process, with bacteria in pairs appearing to be in either chains or clusters.

Table 4.1 Reactions of miscellaneous catalase-negative Gram positive cocci in basic phenotypic tests

Result of phenotypic test		**Morphology**	**Possible identity**
PYR	**LAP**		
+	+	Clusters	*Dolosigranulum pigrum, Facklamia languida, Rothia mucilaginosa, Gemella haemolysans*
+	+	Chains	*Vagococcus, Lactococcus, Ignavigranum, Abiotrophia, Granulicatella, Gemella* spp. other than *G. haemolysins* or *Facklamia* other than *Facklamia languida*
+	-	Chains	*Globicatella, Dolosicoccus*
+	-	Clusters	*Aerococcus viridans, Helcococcus*
-	+	Clusters	*Aerococcus urinae, Pediococcus*
-	+	Chains	Viridans streptococci
-	-	Chains	*Leuconostoc*

Key to Table 4.1. PYR = production of pyrrolidonyl arylamidase, LAP = production of leucine aminopeptidase. Results shown are typical of the majority of strains, reactions of individual strains may vary.
Chains refers to cocci in pairs and chains, clusters refers to cocci that form pairs, tetrads and irregular groups.
Lactococcus strains may show variable reactions in the PYR test.

D. pigrum grows in 6.5% sodium chloride broth but not on bile aesculin, and gives negative results for production of urease, and motility (Laclaire & Facklam, 2000, pp. 2001-2001) but none of these reactions or properties are suitable for consistently identifying cultures of *D. pigrum*. Esculin hydrolysis

can be used to distinguish *D. pigrum* from other catalase negative, PYR and LAP positive organisms, but esculin hydrolysis can be difficult to interpret and it can take up to 14 days to become positive (Hall et al., 2001, pp. 1202-1203). Identification by commercial identification kits is also unsatisfactory. LaClaire and Facklam, (2000b, pp. 2037-2042) evaluated the performance of three commercially produced rapid identification systems for the identification of *D. pigrum*, *Ignavigranum ruoffiae* and *Facklamia* species. All three systems, the bioMerieux rapid ID32 Strep, the BBL Crystal rapid Gram positive identification and the Remel IDS RapID STR (IDS) systems, misidentified the organisms. The bioMerieux API 20 Strep identified *D. pigrum* as *G. haemolysans* (Hoedemaekers et al., 2006, pp. 3461-3462; Lécuyer et al., 2007, pp. 3474-3475) and the API rapidID32 Strep was again found to be unable to identify *D.* pigrum (Lécuyer et al., 2007, pp. 3474-3475). The correct identification can, for the present, only be gained by using a 16S rRNA PCR (Cho et al., 2011, pp. 18-22; Hall et al., 2001, pp. 1202-1203; Hoedemaekers et al., 2006, pp. 3461-3462; Lécuyer et al., 2007, pp. 3474-3475). Because of the difficulty with culture techniques and subsequent identification, investigators have concluded that molecular methods of detection, such as employed in this study, are the future for the study of the numerous catalase negative Gram positive cocci emerging as opportunistic pathogens (Bosshard, Abels, Altwegy, Bottger & Zbinden, 2004, 2065-2073) which includes *D. pigrum* (Cho et al., 2011, pp. 18-22; Lécuyer et al., 2007, pp. 3474-3475). The importance of these bacteria extends to the bacterial toxins hypothesis of MS which posits that a cocktail of transportable antigens from a number of bacteria located in the nasopharynx are responsible for MS (Gay, 2013, pp. 213-232). The catalase-negative, Gram positive cocci

group found in the upper respiratory tract have been reviewed by Ruoff (2002, pp. 1129-1133) and contains a number of viridans streptococci, historically the best recognised members of this large and diverse group of bacteria. All of the viridans streptococci are pyrrolidonyl arylamidase negative and leucine aminopeptidase positive, unlike *D. pigrum* which is pyrrolidonyl arylamidase positive and leucine aminopeptidase positive (Ruoff, 2002, pp. 1129-1133). Most of these streptococci are α-haemolytic on blood agar, leading to their name of viridans, but β-haemolytic and non-haemolytic strains have been described. The salivarius group, *Streptococcus salivarius* and *Streptococcus vestibularis*, are non-haemolytic or α-haemolytic and produce extracellular polysaccharides and are known to cause septicaemia in neutropaenic patients (Ruoff, Miller, Garner, Ferraro & Calderwood, 1989, pp. 305-308). The bovis group are non-enterococcoccal Lancefield group D streptococci that can be divided into many biotypes and, like the salivarius group, produce extracellular polysaccharide-forming bio-types. They can cause septicaemia, endocarditis and also have an association with colonic cancer (Ruoff et al., 1989, pp. 305-308). The mutans group containing *Streptococcus mutans* and *Streptococococcus sobrinus* are found in the oral cavity and are associated with dental caries and endocarditis, and produce extracellular polysaccharides (Coykendall, 1974, pp. 327-338). The mitis group contains *S. pnemoniae*, *Streptococcus mitis* and *Streptococcus oralis* with the former being a recognized pathogen of the lower respiratory tract, but is also responsible for other infections ranging from meningitis to conjunctivitis in susceptible individuals, whilst the latter two are found in infections in neutropaenic patients where *S. mitis* can display penicillin resistance (Doern, Ferraro, Bruggemann & Ruoff, 1996, pp. 891-894). The milleri group of

Streptococcus anginosus, Streptococcus intermedius and *Streptococcus constellatus* may be α-, β- or non-haemolytic and do not produce extracellular polysaccharide but are associated with serious purulent infections throughout the body (Bert, Bariou-Lancelin, & Lambert-Zechovsky, 1998, pp. 385-387). As well as the streptococci there are a number of other catalase-negative Gram positive cocci. *Leuconostoc* and *Pediococcoccus* display intrinsic high level vancomycin resistance and are α- or β- haemolytic (Ruoff, 2002, pp. 1129-1133). Members of both genera are PYR negative and LAP positive (Ruoff, 1999, pp. 306-315). *Leuconostoc* form cocci in pairs and short chains (Handwerger, Horowitz, Coburn, Kolokathis & Wormser, 1990, pp. 602-610), whilst *Pediococcus* form cocci in clusters (Barros, Carvalho, Peralta, Facklam & Teixera, 2001, pp. 1241-1246). Both bacteria have been implicated in septicaemia and other infections in compromised hosts (Ruoff, 1999, pp. 306-315). *Gemella* are α-haemolytic and are associated with endocarditis, meningitis and other infections. There are four species: *Gemella haemolysans, Gemella morbillorium Gemella bergeriae* and *Gemella sanguinis* and have been isolated from a range of infections including endocarditis and meningitis (Facklam & Elliot, 1995, pp. 479-495). *D. pigrum* was originally misidentified as a *Gemella* (Laclaire & Facklam, 2000a, pp. 2001-2003). *Rothia mucilaginosa* can range from catalase positive to negative and is usually non-haemolytic. It is an opportunistic pathogen found in cases of endocarditis and meningitis. *Aerococcus viridans* as the name implies is α-haemolytic and is an infrequent cause of infection in compromised hosts and is PYR positive and LAP negative (Christiansen, Vibits, Ursing & Korner, 1991, pp. 1049-1053). *Aerococcus urinae* can cause urinary tract infections and endocarditis and is PYR negative

and LAP positive (Kawamura et al., 1995, pp. 793-803). *Lactococcus* and *Vagococcus* resemble streptococci, with *Lactococcus* having been isolated from cases of endocarditis. *Globicatella, Facklamia, Ignavigranum* and *Dolosicoccus* have been found in blood cultures (Collins et al., 1999a, pp. 97-101; Collins et al., 1999b, pp, 1439-1442), and *Facklamia hominis* has recently been found in a case of infectious endocarditis (Ananthakrishna et al., 2012, pp. 1-3). The non-haemolytic slowly growing *Helcococcus kunzii* has been described as an agent of wound infections (Woo et al., 2005, pp. 6205-6208) and a new species *Helcococcus sueciensis* has also been found in human wounds (Collins, Falsen, Brownlee & Lawson, 2004, pp. 1557-1560). *Abiotrophia* and *Granulicatella* are Gram-positive cocci in pairs and chains, which are PYR and LAP positive, and are discussed in greater detail in section 4.2. With such a large number of phenotypically similar organisms to *D. pigrum*, and no selective media or reliable biochemical identification systems currently available, the best method to look for *D. pigrum* using bacteriological culture is a rabbit antibody to *D. pigrum* developed by C.W. Gay (2007). This is a polyclonal antibody, and on the evidence of this study the FITC-labelled antibody technique was insufficiently sensitive to detect the presence of *D. pigrum* from clinical specimens, and PCR appears to be the best method for detection and identification of the organism.

Widespread use of PCR has led to revolutionary changes in the phylogeny and taxonomy of bacteria. The use of 16S rRNA sequences for the identification of bacteria has developed dramatically in the last decade and has been particularly useful for the identification of slowly growing fastidious organisms, such as members of the catalase-negative Gram positive cocci group like *D. pigrum*, that

are difficult to grow by conventional bacterial culture methods. Short sequences of 450-650 base pairs are usually sufficient for most identifications, and a large number of bacteria have had their 16S rRNA wholly, or like *D. pigrum*, partially sequenced.

An area of concern with using PCR for this project was the number of samples received in Amies charcoal transport medium. This medium was developed to stop dessication, provide an E_h gradient for anaerobic bacteria, and adsorb out toxins to allow the growth of bacteria during transportation, and there was concern that it might inhibit DNA extraction. However, several publications have concluded that no inhibition occurs using Amies medium for PCR work (Aliyu et al., 2004, pp. 1029-1035; Cloud, Hymas & Carroll, 2002, pp. 3833-3840), and a similar DNA extraction system (Roche MagNA pure) to that employed in this study was successfully used for DNA extraction of *Fusobacterium necrophorum* from Amies swabs by Aliyu and co-workers (2004, pp. 1029-1035).

4.2 Sequence analysis of polymerase chain reaction products

Although the data collected from the sequencing results is too small to draw any definitive conclusions, it is clear that under the running conditions employed the PCR is not entirely specific for *D. pigrum*, as both mixed and non-specific amplification occurs.

Analysis of the 12 sequenced results (Table 3.2) revealed three patterns:-

1) Successful unambiguous amplification of the *D. pigrum* 16S rRNA gene (7/12).

2) Mixed, difficult to distinguish signals (2/12).

3) A clear unambiguous sequence that does not correspond to the *D. pigrum* 16S rRNA gene (3/12).

In the case of the latter, a single amplicon that did not match the *D. pigrum* 16S rRNA was found in B25, A10 and A12. Analysis using the Basic Local Alignment Search Tool (BLAST) of the amplicons showed a remarkable similarity, demonstrating between 100% and 97% 16S rRNA sequence identity with a number of identified (cultured and named) and unidentified sequences (Table 3.3).

Table 3.3 shows that in the three instances of non-*D. pigrum* detection the amplicon produced was consistent. The same sequences with the same accession numbers were identified from the three sequences from three different throat swabs. Amongst the sequences from known sources that with the highest sequence identity was the 16S rRNA gene sequence of *Granulicatella elegans* and the 16S rRNA sequence of *Abiotrophia* species (see Figure 4.1). *Abiotrophia* species were previously known as nutritionally variant streptococci (Frenkel & Hirsch, 1961, pp. 728-730) that exhibited satellitism around colonies of other bacteria. The bacteria are part of the normal flora of the human pharynx, urogenital and intestinal tract but have been implicated in cases of infective endocarditis (Bouvet, 1995, pp. 24-27; Ruoff, 1991, pp. 184-190) and septicaemia and bacteraemia (Christensen & Facklam, 2001, pp. 3520-3523).

G. elegans is a fastidious bacterium that used to belong to the genus *Abiotrophia* (Collins & Lawson, 2000, pp. 365-369) and both it and *Abiotrophia* are taxonomically related to *D. pigrum* (Figure 4.1). It is found in the oral cavity (Ohara-Nemoto et al, 2005, pp. 1405-1407) and has been associated with infective endocarditis (Ohara-Nemoto et al., 2005, pp. 1405-1407; Casalta et al.,

2002, pp. 1845-1847). There are now three other species in the genus *Granulicatella*, *Granulicatella adiacens*, *Granulicatella para-adiacens* and *Granulicatella balaenopterae* (Christensen & Facklam, 2001, pp. 3520-3523; Collins & Lawson, 2000, pp. 365-369) and all of them have been reported as causing infective endocarditis. Christian and Facklam (2001, pp. 3520-3523) identified 25 cases of infective endocarditis (IE) caused by *G. adiacens* and one case by *G. elegans*. Roggenkamp and co-workers (Roggenkamp et al., 1998, pp. 100-104) also reported a case of IE caused by *G. elegans*, and Kanamoto, Sato and Inoue (2000, pp.492-498) reported 45 cases of IE including 15 of *G. adiacens*, 13 of *G. para-adiacens* and eight of *G. elegans*. The genus *Abiotrophia* should now be restricted to *Abiotrophia defective* (Collins & Lawson, 2000, pp. 365-369) and it has been found in nine cases of IE (Kanamoto et al., 2000, pp. 492-498).

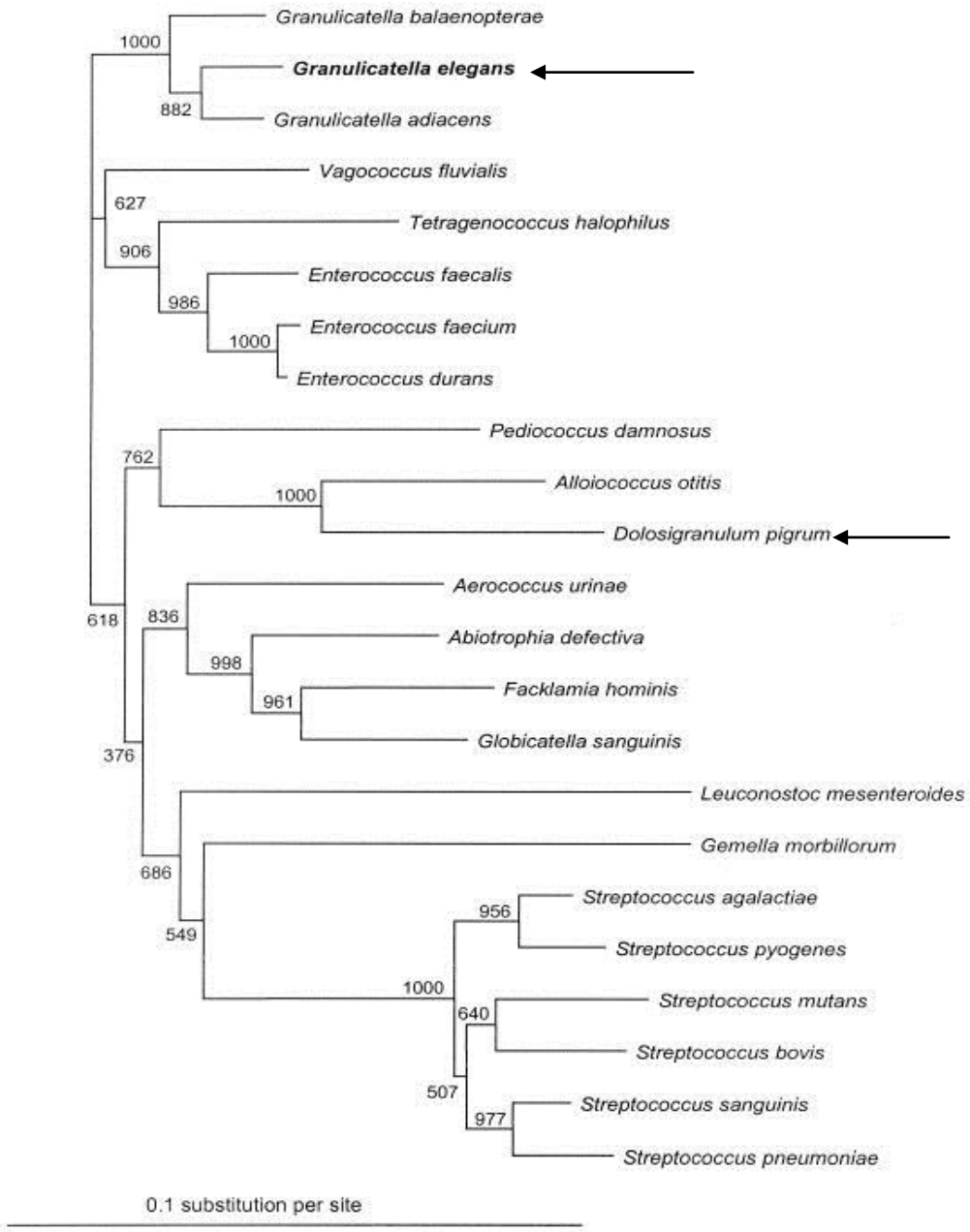

Figure 4.1: Dendrogram representing the phylogenetic relationship between *Granulicatella elegans* and *Dolosigranulum pigrum* (arrowed) among members of the Streptococcaceae.
The tree was derived from a 1,304-bp fragment of the 16S rRNA gene and was constructed by using the neighbor-joining method. Bootstrap values, expressed as a percentage of 1,000 replications, are given at the branching point (Casalta et al., 2002, p. 1846).

In order to establish the reasons for the undesired amplification of non-*D. pigrum* sequences, and to postulate whether their amplification was due to unstable PCR running conditions or non-specific primers, a *G. elegans* rRNA

gene sequence demonstrating 99% homology to rRNA gene sequence B25 was collected and aligned with the known *D. pigrum* 16S rRNA gene sequence. This alignment was then used to compare the *D. pigrum* primer regions to the *G. elegans* rRNA gene sequence:

FORWARD

5′ TAGGTGGATTGGTCGCATGA 3′ *D. pigrum*
5′ TAGGTTCTTGAGTCGCATGA 3′ *G. elegans*
5′ TAGGTTCTTGAGCTGCATGG 3′ *Abiotrophia sp.*

REVERSE (REVERSED AND COMPLEMENTARY)

5′ GGGATCGCTGCTCGTTGTAC 3′ *D. pigrum*
5′ GAGTTCGCTGCTCGTTGTAC 3′ *G. elegans*
5′ GAGTTCGCTGCTCGTTGTAC 3′ *Abiotrophia sp.*

*Shading corresponds to mismatches to the primers designed to amplify *D. pigrum*

Figure 4.2: The *D. pigrum* primer regions.
This is based on the *D. pigrum* 16S gene (X70907) aligned the *G. elegans* and *Abiotrophia sp.* sequences corresponding to A10, A12 and B25 showing some unique *D. pigrum* bases, but with a high degree of 3' similarity.

As can be seen from Figure 4.2 many of the bases in the 3' region of *G. elegans* are identical to *D. pigrum* and could therefore be causing the interference and false positive results. This is a flaw in the primer design as one of the factors in primer specificity is the occurrence of unique bases at the 3' end, because the *taq* polymerase works from 3' to 5', thus preventing attachment and polymerisation of undesired DNA. Developmental work on the primer by Charles Gay as part of an unpublished post-graduate thesis (2009) had demonstrated that the adenine at the 3' end of the forward primer although variable is not unique to *D. pigrum*. It is possible, therefore, that the PCR might not be specific in the few instances in which adenine *does* exist in this position in other species.

Analysis of the sample types demonstrated some trends

Naso-pharyngeal aspirates

Five of the NPA amplicons corresponded to *D. pigrum*, demonstrating a single well matched amplicon. C17 gave mixed signals, suggesting that more than one gene was amplified, or that mistakes were made during the polymerase reaction (poor PCR fidelity).

Nose swabs

The two amplicons that were sequenced from this source demonstrated an amplicon that matched the *D. pigrum* gene. One sequence, A20 was not very high above the background, suggesting that there could have been some low level amplification of other gene sequences, or mistakes during the polymerase reaction (poor PCR fidelity).

Throat

None of the four throat amplicons revealed a *D. pigrum* sequence. Three of them had a clear signal, but this did not correspond to *D. pigrum*. The other sequence, A8, demonstrated mixed signals, again suggesting that more than one gene was amplified, or that mistakes were made during the polymerase reaction (poor PCR fidelity).

Each type of sample comes from sites with very different environments containing different bacterial flora and therefore different DNA. For the molecular detection of *D. pigrum* the oropharyngeal region appeared to be the most problematic with no *D. pigrum* detected but *Abiotrophia* species, and particularly *G. elegans*, posing a problem for the primers used. These bacteria are not normally found in the nasal or nasopharyngeal regions (Ruoff, 1991, pp. 184-190; Bouvet, 1995, pp. 24-27), and so although the primers were not ideal,

the fact that they contained *D. pigrum* specific bases that may be sufficient for specific amplification, means that they may be utilized to screen for *D. pigrum* in nasal and nasopharyngeal specimens. Carriage of *D. pigrum* is of particular interest from these sites because of the link found by Gay and co-workers between MS and sinusitis (Gay et al.,1986, pp. 815-819; Dick and Gay, 1988, pp. 25-35) leading to the bacterial toxins hypothesis of the cause of MS (Gay, 2007, pp. 105-112).

A further study is necessary to fully evaluate the PCR. During the development of the PCR as part of an unpublished post-graduate thesis (Gay, 2009) it was uncertain if 30 cycles should be used instead of 35 cycles, as using a relatively high number of cycles (35) can lead to polymerase activity saturation, which is known to be a cause of a high level of mistakes during the elongation step. This can lead to the erroneous results and non-specific amplification seen in this study. However, 30 cycles could lead to a crucial loss of sensitivity and the failure to detect small numbers of *D. pigrum* and so it was decided to use 35 cycles. A solution to this problem could be to use a two step (nested) technique which would maximize the sensitivity whilst maintaining the specificity of the PCR. This has been used in detecting *Histoplasma capsulatum* in human tissue (Bialek et al., 2002, pp. 1644-1647). Raising the annealing temperature by a degree or two should also be attempted as this may help to increase the specificity, which could be crucial in the early stages of the PCR, helping to eliminate non-specific amplicons. Therefore, an adjustment to the crucial ratios of primer to template and polymerase to template may result in an improved specificity, and a further study comparing the PCR with both running conditions is indicated to ensure that the sensitivity is not compromised. If this does not

yield acceptible results, then the primers will have to be redesigned so that a PCR possessing high sensitivity and specificity is produced which can be used to screen for *D. pigrum* in clinical specimens. The problem is that to date the 16S rRNA is the only part of the *D. pigrum* genome that has been sequenced. This makes designing a species specific PCR difficult as many other species will have closely homologous sequences, so the task of incorporating *D. pigrum* specific bases into highly specific primers is made much more difficult. The future thus relies upon the sequencing of the whole genome of *D. pigrum* so that there is a better chance of designing a highly specific primer.

4.3 Detection of *Dolosigranulum pigrum*

From the sequencing results it would appear that any PCR product with a single band at 1085 bp is amplifying *D. pigrum*, but any product producing a double band is producing mixed amplicons or detecting *Abiotrophia* species or *G. elegans*. If an assumption is made that this is also true of the other PCR results that were not sent for sequence analysis then in total there were nineteen positive specimens out of eighty eight (21%). There were twelve males to seven females, ranging in age from seventy three years to 8 days in age, with the majority being under a year old (11, 58%) indicating that acquisition of *D. pigrum* occurs at a very early age. The majority of samples matching with the *D. pigrum* gene were naso-pharyngeal aspirates (10, 55%), with seven from throat swabs and two from nose swabs. This is consistant with LaClaire and Facklam's findings in 2000 (pp. 2001-2003) (Table 1.1) which detected the bacterium in respiratory samples including naso-pharyngeal specimens. The results of the microbiological testing of the samples before they were used in this

study were accessed. This gave some idea of the microbial flora present, although it was an incomplete picture as non-pathogenic organisms were reported as "normal flora" or "skin flora", or not fully identified and reported as "coliforms". There appeared to be no particular association with recognized pathogens, with six samples matching with the *D. pigrum* gene containing pathogens and thirteen no pathogens. It would be interesting to perform another study using the *D. pigrum* probes from this study and fully identifying and characterizing any bacteria found, especially from nasal and naso-pharyngeal samples, which would lead to a greater understanding of the microbial communities found in the upper respiratory tract. The current study indicates that *D. pigrum* is present from an early age (Table 3.1), though whether it is causing disease or is present as a permanent or transitory part of the normal flora can not be said. Recent evidence supports these findings with Laufer and co-workers finding *D. pigrum* in nasal swabs of children aged 6 to 78 months in a study on the bacterial flora of the nasal cavity (Laufer et al., 2011). The study concluded that *D. pigrum* was a member of the normal flora and imparted a protective effect against colonization leading to otitis media from *S. pneumoniae* in children.

4.4 Enzyme linked immunosorbent assay and Western blotting

The development of ELISA tests to look for antibody has been used successfully to look for equine influenza virus (Denyer, Crowther, Wardley & Burrows, 1984, pp. 609-620), and the use of ELISA followed by Western blotting for antibody investigations are a proven method of investigating diseases, and have been used as an algorithm for HIV testing ("HIV Assays…", 2004, pp. 9-15), and for published studies (Figueroa et al., 2000, pp. 57-62).

In this study there was a statistically significant difference in antibody levels to *D. pigrum* in the MS group compared with age and sex matched controls. Western blotting of 15 of these sera failed to detect bands common to *D. pigrum*. Raised intrathecal and blood antibodies to a number of organisms have been reported in MS (Salmi, Reunanen, Ilonen & Panelius, 1983, pp. 241-249; Salmi, Viljanen & Reunanen, 1981, pp. 333-341; Vartdal, Vandvik & Norrby, 1980, pp. 248-255) but these findings were not uniformly accepted as other studies failed to find a significant difference between MS and controls (Norrby, 1978, pp. 1-39; Vandvik & Norrby, 1989, pp. 769-771). It is possible that the failure to find bands common to *D. pigrum* on Western blotting may be due to a lack of sensitivity caused by using the culture supernatant of whole cell *D. pigrum*. Equally the findings of raised antibody levels in this study may indicate that antibodies to *D. pigrum* are a biological marker suggestive of leakage of bacterial products from the upper respiratory tract into the central nervous system which may initiate MS. Indeed, using isoelectric focusing with antigen imprinting to investigate oligoclonal immunoglobulin bands (OCBs) in MS patients, Jorge (1995), found evidence of reaction between OCBs and the supernatant of cultures of staphylococci. The origin of OCBs found in MS has never been explained although they are not directed to any viruses that have been studied in conjunction with MS or to CNS antigens (Mehta, Patrick, Mehta & Wiseniewski, 1987, pp. 746-751). This has led to them being dismissed as 'nonsense antibody' without any antigenic specificity (Patersson & Whitacre, 1981, pp. 111-117). The finding in this study that systemic levels of antibody are elevated in MS raises the question could the OCBs in MS be formed in response to bacterial toxins leaking into the CNS.

Transportable products, such as toxins, made by Gram positive bacteria demonstrate cross reactivity across species (Dinges, Orwin & Schlievert, 2000, pp. 16-34) and such cross reactivity has been found with staphylococcal enterotoxin B (sphingomyelinase) and a 28 kDa *D. pigrum* protein (C.W. Gay, 2007) (Figure 4.3).

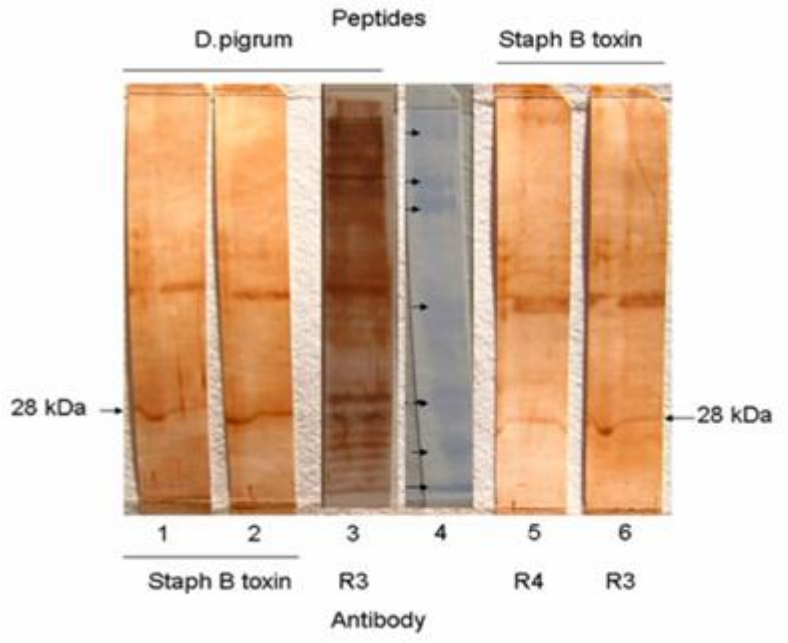

Figure 4.3. A cross reactive 28 kDa band between *Staphylococcus aureus* and *Dolosigranulum pigrum*. Demonstrated using anti-*Staphylococcus aureus* enterotoxin B antibody against *Dolosigranulum pigrum* peptides (lane 1, 2), and anti- *Dolosigranulum pigrum* antibody (rabbit R3 and R4) against *Staphylococcus aureus* enterotoxin B (lanes 5, 6). (C.W. Gay, 2007).

This *D. pigrum* protein may be a sphingomyelinase, as this toxin is associated with hot/cold haemolysis, something which *D. pigrum* demonstrates (Figures 1.6 and 1.7),

As mentioned it seems likely that any such involvement of bacterial toxins in MS will be polymicrobial, and in the light of the well documented carriage of *S. aureus* in the nasal tract (Kluytmans, van Belkum & Verbrugh, 1997, pp. 505-520) and Gay's (2013, pp. 213-232) finding using immunocytology of an IgG from one of

the rabbits(R3) detecting antigen ('R3.89') in association with early primary MS lesions. Evidence from mass spectroscopy suggested that the antigen was staphylococcal neutral sphingomyelinase (Gay, 2013, pp. 213-232). Antibody to R3.89 was also detected in the other rabbits (R4, R5 and R6) at lower titres (D. Gay, personal communication, February 04, 2009). Many Gram positive bacteria, produce these toxins which have been associated with neurological diseases (Uyeda, Gerstl, Smith & Carr, 1966, pp. 143-146). Sphingomyelinase hydolyses the phosphodiester bond of sphingomyelin in target cells and the susceptibility of these cells appears to be directly related to the sphingomyelin content of their cell membrane. Sphingomyelinase from external sources, such as from bacteria, results in the release of ceramide as a result of extensive endocytosis of plasma membranes (Zha et al., 1998, pp. 39-47). This release of ceramide triggers the export of internal cellular sphingomyelinases to the outer surface of the cellular plasma membrane leading to more sphingomyelin being hydrolysed in a chain reaction which results in the initiation of cell apoptosis (Rebillard et al., 2007, pp. 7865-7874). Oligodendrocyte apoptosis has been induced in cell culture using staphylococcal neutral sphingomyelinase (Jana & Pahan, 2007, pp. 184-193) mediated by ceremide (Lee et al., 2004, pp. 123-131), and Barnett and Prineas (2004, pp. 458-468) have proposed that oligodendrocyte cell death is a primary event in early MS and occurs in pre-demyelinating lesions. Whilst this may be true, and oligodendrocytes certainly demonstrate a high susceptibility to neutral sphingomyelinases, it is unlikely that they are the sole mediators for the development of MS. It is more likely that they are one of a number of transportables produced by a variety of species within the flora of the nasopharynx which act together to manipulate the host immune response. Evidence for this happening has been seen with the

staphylococcal toxic shock syndrome toxin (TSST-1), where the superantigenic mitogenic signal of the toxin has been augmented by the presence of sphingomyelinase (Huesby et al., 2007, pp. 8719-8726). It is this pathogenic heterogeneity which makes it difficult to establish which are the key toxins responsible for the development of MS.

4.5 Future work to validate the bacterial toxins hypothesis

Although the primers for the PCR used for this thesis need refining, this study found *D. pigrum* in the nasal tract and nasopharynx. From this location the bacteria may be able to gain entry to the sinuses, and from there export toxins into the CNS. Further work is indicated to establish the composition of the bacterial flora of the nasopharynx in MS, as the toxins which intiate the disease may be polymicrobial. Such studies should be performed using a matched control group to see if there are any differences in the bacterial flora in MS, and should include MS patients at different stages of their disease to see if there is any difference in the composition of the flora in early stages of the disease. Molecular studies, using a panel of primers from the 16S rRNA of bacteria, should be included as there are a number of fastidious bacteria, including the catalase negative Gram positive cocci found in the nasopharynx, which are difficult to culture on bacteriological media (Casalta et al., 2002, pp. 1845-1847). A recent study on the bacterial flora of normal adult nasal cavities highlighted the benefits of using molecular methods (Cho et al., pp. 18-22). The researchers used bacteriological culture methods and polymerase chain reaction-denaturating gradient gel electrophoresis (PCR-DGGE) of 16S rRNA gene fragments on 19 nasal swabs. On culture only nine species were detected, with *Staphylococcus epidermidis* being the most frequently detected

followed by *Staphylococcus aureus*. The detection rate of the other bacteria was very low. In contrast using PCR-DGGE 23 different kinds of bacteria were detected of 9 genera: *Staphylococcus, Dolosigranulum, Bacillus, Corynebacterium, Enterobacter, Actinobacterium, Hafnia, Moraxella and Clostridium*. *Staphylococcus* species and *Enterobacter aerogenes* were isolated most frequently. Staphylococci have recently been implicated in MS (Gay, 2013, pp. 213-232). It is known that 30-40% of *S. aureus* isolates from human infections are β-toxin positive, with nasal carriage in the general population of 11% (Hedström & Malmqvist, 1982, pp. 217-220). Furthermore some strains produce an incredible 500 μg toxin per ml in culture (Huesby et al., 2007, pp. 8719-8726) and this raises the possibility that particular toxigenic strains of *S. aureus* may be specifically associated with MS. Mulvey and co-workers (2011, pp. 397-403) have used genetic techniques to identify enterotoxigenic strains of *S. aureus* in MS patients and the nasal flora of MS patients should now be examined for high secreting toxigenic strains. Such work may lead to the exciting development of a specific laboratory test for MS. Elevated serum antibody levels were found in this study in MS patients, but they were not found to be specific for *D. pigrum*. This may be a sensitivity issue as the Western blotting used supernatant from whole cell culture of *D. pigrum*. Further research of *D. pigrum* would enable specific purified specific toxins to be used to establish if there is an elevation of anti-*D. pigrum* in MS. The creation of an ELISA test for screening MS CSF for anti-*D. pigrum* is also indicated. Further genetic sequencing of *D. pigrum* should proceed to enable specific primers to be made both for further identification and toxin studies, which would help to prove if *D. pigrum* were involved in MS. Such genetic information could also be used in studies to see if the sequences for the toxins are analogous

with the the exotoxins of other bacteria. This is pertinent as, already discussed, *D. pigrum* may produce a sphingomyelinase which cross-reacts with staphylococcal sphingomyelinase, a bacterial toxin that has recently been proposed as a possible instigator of MS (Gay, 2013, pp. 213-232). A further study employing an ELISA to look for anti-sphingomyelinase in MS in serum and CSF is therefore indicated. Sphingomyelinase could be looked for using PCR in 'nose blows' or nose swabs. This technique could also be used to screen MS CSF for the presence of bacterial products. The intranasal inoculation of transportable toxins such as staphylococcal sphingomyelinase and enterotoxins thought to be involved in the immune dysregulation of MS using animal models such as rats should also take place. Such studies on bacterial products or toxins might go some way to seeing if the bacterial hypothesis of MS is valid or not.

Finally the use of a radiographic marker into the nose, and subsequent tracking of its progress using magnetic resonance imaging could establish a potential route of access of bacterial products behind the blood-brain barrier, and directly to the CNS which would help to validate the bacterial toxins hypothesis. If such a route is proven then it would also allow the entry of therapeutic drugs for a number of neurological diseases, including MS, directly into the CNS (Wen et al., 2011, pp. 131-138; Gay, 2012, pp. 154-155). This may have implications in MS as it has been argued that early treatment of respiratory bacterial infections in the disease may have a beneficial effect (Herrmann et al., 2006, pp. 4841-4848), although the use of penicillin was found not to be associated with a decreased risk of MS (Alonso, Jick, Jick & Hernan, 2006, pp. 997-1002).

4.6 Conclusions

The main conclusions from the study were:

- An ELISA to screen for antibodies to *D. pigrum* in the serum of 65 MS and matched controls was successfully designed and run. Levels of detectable IgG associated with *D. pigrum* in the MS group were found to be significantly different than the control group, $p <= 0.001$.
- Using Western blotting 15 of the sera demonstrating detectable antibody levels in their sera in the ELISA, along with their matched controls were run against *D. pigrum* NCIMB 702975 culture supernatant. There was a lack of common bands between the MS sera and with R6 rabbit anti-*D. pigrum* sera from a previous study (C.W. Gay, 2007), but this may be due to the fact that supernatant of whole cell *D. pigrum* was used in the current study. Research into *D. pigrum* toxins and sequencing of the bacterium in its entirety would provide opportunities to further test an association between *D. pigrum* and MS.
- Eighty eight respiratory, eye and ear swabs and various body fluids were screened for *D. pigrum* by culture and anti-*D . pigrum* (R3) / fluorescein isothiocyanate microscopy. All were negative. These findings concur with other studies that conclude that the detection of the bacterium should be performed by molecular means using PCR.
- The PCR used in this project successfully detected *D. pigrum* in nasal and naso-pharyngeal specimens, proving that the bacteria were in the right location to be able to leak toxins into the CNS in MS. The PCR was less successful in other sites, especially in the oropharynx where the closely related *G. elegans* and *Abiotrophia* gave false positive reactions.

- The bacterial toxin hypothesis was not disproved, *D. pigrum* being found in the nasopharynx where, anatomically toxins from it could enter the CNS to instigate MS. A significant elevation of anti-*D. pigrum* was detected in the sera of MS patients when compared with a matched control group, and although these antibodies could not be linked specifically to MS by Western blotting further work on sequencing *D. pigrum* could lead to the development of primers capable of screening for toxins and antibodies to these and other bacterial products, which may provide evidence of a specific association between *D. pigrum*, and other bacteria found in the nasopharynx, and MS. If specific bacterial products linked to MS are thus identified then they could be used to develop ELISA tests to look for antibodies or, more excitingly, the bacteria could be looked for directly using a PCR test. This test could be performed on non-invasive samples such as 'nose blows'. The development of such a molecular test could lead to specific and early diagnosis, and possibly treatment, of the disease using, for example, specific monoclonal antibodies, before serious damage occurs to the brain and spinal cord.

Chapter 5:
Reflections on the Professional Doctorate

Professional doctorates (PDs) are not new and the first professional practice doctorate was granted in Paris in the 12th century (Bournier, Bowden & Lang, 2001, pp. 65-83) many years before the first PhD in 1861 (Bournier, Bowden & Lang, 2000, pp. 226-237). What drew me towards the professional doctorate was the fact that it could be studied part time and I therefore would not have to take a career break to complete it. I saw it as a course that would gradually increase my skills in a journey of further and continuous development rather than a means to a fixed goal as a PhD would have been. Although an increasing number of PhDs contain taught elements like PDs, these tend to be individualized based study programmes rather than cohort-based as is found with PDs. This is an important issue as the support I found from the lecturing staff and classmates was integral to developing as a researcher and practioner in the difficult first few years of a doctoral programme. It was therefore traumatic to discover in Part 2 of the PD that this level of support was no longer available. Despite being used to being self directed this was a difficult period, and the disadvantages attempting to perform research part time was magnified by the problems of Government induced change in the form of Transforming Pathology and by personal tragedy. I found the research project challenging, but developing the skills to plan, set up and manage a multi-site research project, plus the experience of gaining ethical approval, will directly benefit me when I undertake further research, and also enable me to offer guidance to colleagues in the future. Having personally obtained all the research monies from a mixture of Institute of Biomedical

Science (IBMS) grants, research awards and charitable donations I have become more aware of sources for research funding. I have also during the PD become a budget holder for these research monies which financed related research projects of a small group of researchers, including the MSc research project of Charles Gay which developed the PCR probes I utilised for my study at the University of Essex. Without this developmental work I would have been unable to include the PCR part of the study because of the time restraints of conducting research on a part-time basis. An article in the local newspapers, arranged through Colchester Hospital University Foundation Trust's Head of External Relations, Mark Prentice, reported that I had been awarded a research grant to research multiple sclerosis. This led to a local businessman contacting me to ask if he could help to purchase equipment for the research as he felt that the amount that I had been awarded was too small to research such an important subject. It transpired that a friend of his had contracted multiple sclerosis and had been deteriotating rapidly, and he was desperate to fund research into the disease. I arranged for the money to be used as a charitable donation which enabled an expensive microscope camera to be purchased. This has been used extensively by the research group for photographs in a number of published papers, the latest of which (Gay, 2013, pp. 213-232) gave an acknowledgement to me for bacteriological and administrative help. Using the administrative skills I have gained in planning and setting up my doctoral project I have assumed responsibility for the receipting of goods for the Microbiology Department, Colchester, and have written a database for the recording of standing orders, as well as becoming a signatory for non-stock requisition ordering for the department.

In general it is widely held that a PhD is designed to prepare the student for a career in academia by breadth and originality of research, whilst a PD, although equal in rigour, is intended to further the career of experienced practioners in a specific field. To this end a PD research project tends to be problem focused and concentrated on the application of theory and knowledge to a particular profession, whilst a PhD project is involved in trying to create new theories or knowledge. Hambrick (1997, pp. 133-148) has argued that for a professional doctorate to educate only for practitioner roles is too limiting. Certainly my project is not a typical professional doctorate project as it is not based on the premise of answering a problem in my practice. It has elements of a PhD as it is involved in furthering the knowledge of MS and has elements of originality as it proposes a new hypothesis for the pathogenesis of this little understood disease (Jorge, 2009a, pp. 573-577). Yet it also has elements of a PD project as it addresses professional practice problems in medical microbiology such as the optimal method of isolating and identifying slow growing potential pathogens such as *D. pigrum*, which can pose a significant clinical problem. The creation of novel enzyme linked immunosorbent assays (ELISA) and polymerase chain reaction (PCR) tests for the project also helped me to understand these procedures which are becoming increasingly important as medical microbiology moves from traditional manual cultural methods to automated techniques, and from biochemical identification to use of molecular biology and mass spectrometry to characterise and speciate micro-organisms. I now feel better able now to impart knowledge and understanding of these new techniques that are rapidly shaping the provision of the services of medical microbiology in the 21st century to other microbiology staff members.

This understanding, and the skills I have gained from the taught modules of the PD, have thus not only helped me directly as a practitioner but with training and educating microbiology staff and students from the University of Essex, something which I have become more active in since I started the PD. I now lecture and run practicals for undergraduates at the University of Essex on the biomedical sciences course, and have helped to set up their MSc course, on which I give lectures on the microbiology units, and set questions for the final examinations. I have also begun running a 'summer school' week for the undergraduates in pathology and mark their assessed essays, reflective diaries and presentations.

Another area that the PD has improved me as a practitioner is in publication and dissemination. Prior to beginning the course I had not considered publication, but inspired by the PD I published two articles (Jorge, 2009a, pp. 573-577; Jorge, 2009b, pp. 125-126) and will look to write more. The first of these articles led to an invitation to give a presentation to the London and South East Region (LASER) conference "*Brave New Workforce"* in Brighton on the 4th and 5th June 2009, where I explained about my project, and argued for the usefulness of PD, detailing my experiences and research project. I concluded that it was a worthwhile degree for the individual development of experienced biomedical scientists with benefits for the practitioner, profession and employers. Whilst at this conference I met Keith Kitson who had been writing on critical reflection in biomedical science (Kitson, 2005) and this inspired me to regularly lecture on the subject to the University of Essex undergraduate students. One of the problems cited at a recent biomedical science meeting at the University of Essex was the difficulties some students had with the reflective diaries needed for their

portfolios and I have expressed an interest in lecturing and running workshops on this subject for the students.

Although my research project does not prove the bacterial toxin hypothesis it does not disprove it. The complete sequencing of the genome of both *D. pigrum* and staphylococci will enable the production of specific primers to look for the presence of the bacteria and their toxins in multiple sclerosis. This could be done using PCR on both serum and CSF in case control studies. Animal experiments using a range of toxins should also be carried out, and markers using, for instance, current therapeutic agents, added to the nose of human volunteers, both MS and matched controls, and tracked using magnetic resonance imaging could also be used to establish if the bacterial toxins hypothesis is valid. I hope that my work will be used to continue research into MS and the bacterial toxins hypothesis. For this to happen it is important to disseminate my results. To this aim I have given a series of four presentations to the microbiology department. The first presentation outlined the history of MS and explained what the disease is and current thoughts on diagnosis and treatment. The second examined current hypotheses on the cause of MS and explained the bacterial toxins hypothesis on which my current research is based. The third explained how PCR works and the results from my project and the fourth explained the principles of ELISA and Western blotting and detailed the results from my project. I also hope to publish my doctoral research in a peer reviewed journal such as the *British Journal of Biomedical Science*. I have also been contacted by Mark Prentice again, who interviewed me and a member of the research team, Derek Gay, about including news of our research into MS in an internal newsletter, Mainstream. Mark was particularly interested in the history of the research as it

all began in microbiology in Colchester in 1986 when Derek started work as an independent researcher here. It was also agreed to put out a press release to the local newspapers to coincide with the publication of Derek Gay's paper in the scientific journal *Multiple Sclerosis and Related Disorders* (Gay, 2013, pp. 213-232) about the MS research that had taken place using monies won as a research prize from Colchester Hospital University Foundation Trust. Mark was particularly keen to stress the fact that I worked for the trust, and to highlight the research for my PD. This publicity and the publication of my research findings, and Derek's paper, will promote the hypothesis of a bacterial cause of MS, and will hopefully lead to more research that may lead to a diagnostic test for MS and an effective prophylaxis and/or treatment for this dreadful disease.

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Appendix A: Ethics, research grants and awards

A1 Ethics

Essex 2 Research Ethics Committee
Terminus House
9th Floor
The High
Harlow
Essex
CM20 1XA

Telephone: 01279 419312
Facsimile: 01279 694917

10 February 2008

Mr Mark Francis Jorge
Senior Biomedical Scientist
Essex Rivers Healthcare Trust
Microbiology Department
214 Turner Road
Colchester
CO4 5JR

Dear Mr Jorge

Full title of study: **The potential pathogenicity of Dolosigranulum pigrum in Multiple Sclerosis, and the occurance of the organism in the upper respiratory tract.**
REC reference number: **08/H0302/4**

Thank you for your letter of 24 January 2008, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair in consultation with the lead readers for your study.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised.

Ethical review of research sites

The Committee has designated this study as exempt from site-specific assessment (SSA. There is no requirement for [other] Local Research Ethics Committees to be informed or for site-specific assessment to be carried out at each site.

Conditions of approval

The favourable opinion is given provided that you comply with the conditions set out in the attached document. The committee also request that you notify the coordinator of the registration number for the Registered Tissue Bank which will be used to supply tissue for this study. You are advised to study the conditions carefully.

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*
Application	Version 5.5	11 December 2007
Investigator CV		10 December 2007
Protocol	Version 5.0	23 January 2008
Covering Letter		10 December 2007
Summary/Synopsis	Version 3.0	04 December 2007
Letter from Sponsor		27 November 2007
Response to Request for Further Information		24 January 2008
CV Dr D R Memagh Supervisor		28 November 2007
Risk Assessment Dept Biological Services		
Dept of Microbiology COSHH		
Employee Risk Assessment Form R024		
General Risk Assessment Form R50		
General Risk Assessment Form R023		
Emails from Queries Line NRES		05 June 2007

R&D approval

All researchers and research collaborators who will be participating in the research at NHS sites should apply for R&D approval from the relevant care organisation, if they have not yet done so. R&D approval is required, whether or not the study is exempt from SSA. You should advise researchers and local collaborators accordingly.

Guidance on applying for R&D approval is available from http://www.rdforum.nhs.uk/rdform.htm.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Now that you have completed the application process please visit the National Research Ethics Website > After Review

Here you will find links to the following

a) Providing feedback. You are invited to give your view of the service that you have received from the National Research Ethics Service on the application procedure. If you wish to make your views known please use the feedback form available on the website.
b) Progress Reports. Please refer to the attached Standard conditions of approval by Research Ethics Committees.
c) Safety Reports. Please refer to the attached Standard conditions of approval by Research Ethics Committees.
d) Amendments. Please refer to the attached Standard conditions of approval by Research Ethics Committees.
e) End of Study/Project. Please refer to the attached Standard conditions of approval by Research Ethics Committees.

We would also like to inform you that we consult regularly with stakeholders to improve our service. If you would like to join our Reference Group please email referencegroup@nationalres.org.uk .

08/H0302/4	**Please quote this number on all correspondence**

With the Committee's best wishes for the success of this project

Yours sincerely

Mr Jon Gould
Chair

Email: suzanne.emerton@eoe.nhs.uk

Enclosures: Standard approval conditions

Copy to: Dr Graham Mills
University of Portsmouth
School of Pharmacy & Biomedical Science
Room 2
25 St Michaels Building
White Swan Road
Portsmouth
PO1 2DT

Essex Rivers Healthcare Trust R & D office

Essex Rivers Healthcare Research and Development Reference No: 2007/038

The Research and Development Steering Group gave approval for the project on the 14th February 2008.

Dear Mark,

Ethical approval has been granted and work can proceed, but would you please send another couple of signed copies (pages 1 and 2) of the University form as before.

Thank you,

Terry

Dr. Terry J. McGenity
Department of Biological Sciences,
University of Essex, Wivenhoe Park,
Colchester, CO4 3SQ, UK
T. +44 (0)1206 872535, F. +44 (0)1206 872592
email TJMcGen@essex.ac.uk

A2 – Research grants and awards

Serving the people of north east Essex

Colchester Hospital University NHS
NHS Foundation Trust

Colchester General Hospital
Research and Development Office
Postgraduate Medical Centre
Turner Road
Colchester
CO4 5JL

Tel: 01206 744242
Fax: 01206 851231
E Mail: research@colchesterhospital.nhs.uk

Mark Jorge

Dr F Gay
Hon Consultant Pathologist
Cambridge Biostability
Cambridge Science Park
CB4 0GA

9th June 2008

Dear Dr Gay,

Re: Research Prize Award – An investigation into the role of bacterial transportable myelinases (beta haemolysins) in the induction of demyelination in multiple sclerosis

Thank you for the more detailed submission of the proposal. Following further consideration by the Research & Development Steering Group it has been decided that the award will be split between two projects.

Therefore, I am pleased to inform you that the above project has been awarded £14,000. Please could you provide the R&D Office with details of where the funds should be paid into i.e. Bank account name, number and sort code.

The Steering Group would like to pass on their congratulations and wish you every success with the project. Please do keep the R&D Office informed of your progress.

Yours sincerely

Dr R A Elston
Director of R&D

Cc Mr Mark Jorge, Senior Biomedical Bioscientist
Dawn Worsfold,Finance

Institute of Biomedical Science
12 Coldbath Square, London EC1R 5HL
Tel: 020 7713 0214 • Fax: 020 7837 9658
E-mail: mail@ibms.org
Website: www.ibms.org

CHIEF EXECUTIVE
Alan R Prior
MBE MPhil CSci FIBMS

SM/mhj

8 July 2008

Mr M F A Jorge CSci FIBMS
Microbiology Department
Essex Rivers Healthcare Trust
214 Turner Road
Colchester
CO4 5RJ

Membership No: 10812

Dear Mark

Research Grant Application – 2008

Following consideration by the Professional Examinations and Awards Committee, I am pleased to advise that you have been awarded a grant of £3,800.00 to assist you with the consumables for your research project. For security and speed the Institute makes all payments electronically directly into the bank account of the recipient I would therefore ask that you send my assistant Marie-Helen, at marie-helenjean@ibms.org, the information detailed below so that disbursement of the grant can be arranged:

- Bank name and full address
- Bank sort code
- Account name
- Account number

Within 12 months from the date of this letter you must submit to the Institute:

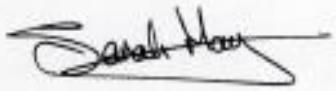
- A schedule of the items on which the grant was spent;
- A progress report on the research and;
- A listing of the publications in which the results of the research have been or will be featured.

A further condition of this award is that any proposed publication must be offered to the British Journal of Biomedical Science. Due acknowledgement of the Institute's research grant should be made in any published work. Manuscripts arising from supported research projects may be entered for appropriate prizes awarded to Institute members.

I would be grateful if you could possibly send me, a photograph of yourself and a paragraph or two about the project so that it can be featured The Biomedical Scientist. I would prefer if you could email both the picture and text to my assistant Marie-Helen at the email address above.

I would like to take this opportunity to wish you every success with your project.

With kind regards



Sarah May
Deputy Chief Executive

Appendix B: Reagents and materials

B.1 Reagents for DNA extraction using PureLink DNA kits from Invitrogen (K1820-01)

PureLink Genomic Lysis/Binding Buffer

Lysozyme (Sigma, L6876)

PureLink Genomic Digestion Buffer

PureLink Proteinase K (20 μg ml^{-1})

PureLink Wash Buffer 1

PureLink Wash Buffer 2

PureLink Elution Buffer

B.2 Electrophoresis Reagents and materials

Agarose (Fisher Scientific BP1356-100)

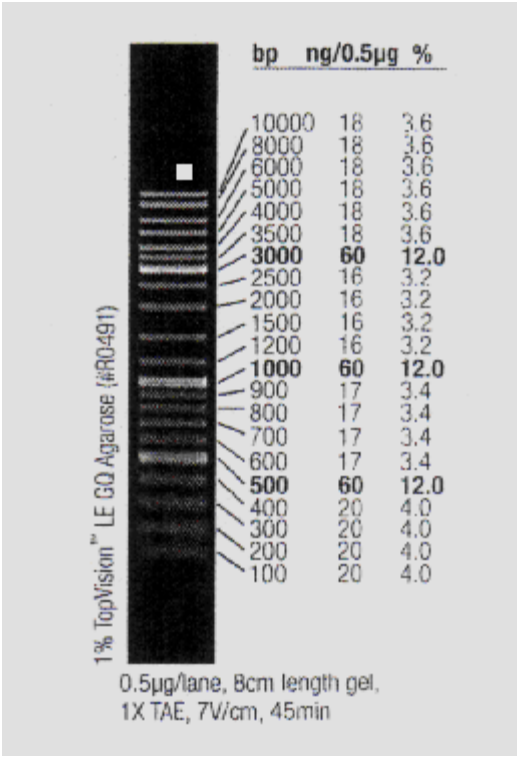
Tris-Acetate- EDTA (TAE) buffer for agarose:
TAE x 50 stock solution, containing:
Tris base, 121 g
Glacial acetic acid, 28.55 ml
0.5 M EDTA (pH 8.0). 50 ml

Loading dye for gel electrophoresis (Fisher Scientific)
10 mM Tris-HCl (pH 7.6)
Bromophenol blue, 0.03% w/v
Xylene cyanol FF, 0.03% v/v
Glycerol 60% v/v
EDTA. 60 mM

DNA ladder, Fermentas, GeneRuler SMO333

TAE buffer.
242 g TRIS base, 57.1 ml glacial acetic acid, 100 ml 0.5 ml EDTA, (pH 8.0) made up to 1 litre.

B.3 Gene Ruler DNA Ladder Mix



Fermentas Life Sciences. #SMO333.
This shows the relative positions of the different size DNA reference fragments. This DNA ladder has been used for all the experimental gels.

B.4 *Dolosigranulum pigrum* ELISA reagents and materials

Rabbit 6 anti-*D .pigrum* pre and post immune serum

Chicken egg albumin – Sigma A-5503

BBL FTA haemagglutination buffer (Phosphate buffered saline pH 7.2 +/-

0.1) - Becton Dickinson 211248

Brain Heart Infusion – Oxoid (CM0225)

Tetramethylbenzadine buffer/chromagen (TMB) - BioRad 492430

Anti-Rabbit IgG (whole molecule) peroxidase conjugate – Sigma A6154

Anti-Human IgG (γ-chain specific) peroxidase conjugate – Sigma A6029

Stopping solution (1 N sulphuric acid)

Sterilin Microtitre Plate (96 well, flat bottom – 611 F96)

Sterilin polystyrene round bottom tubes (75 x 13 mm – 30924)

Triturus 4 plate robotic EIA analyser – Grifols USA

B.5 Western Blotting Reagents and materials

Mini-Protean II dual slab cell – Bio-Rad

Goat anti-human IgG – Sigma Aldrich 18885

Anti-goat horse radish peroxidase – Sigma Aldrich 12136

Diaminobenzidine (DAB) enhanced liquid substrate system for membrane ELISA – Sigma Aldrich D6815

Nitrocellulose membranes O.45 microns - Sigma-Aldrich N8267-5EA.

Pre–cut filter paper - Bio-Rad 170-3932

Acrylamide - Sigma Aldrich A3553

Tris(hydroxymethyl)aminomethane – Sigma Aldrich 252859

Sodium dodecyl sulphate - Sigma Aldrich L3771

Mercaptoethanol - Sigma Aldrich M3148

Ammonium persulphate - Sigma Aldrich A3678

Methanol - Sigma Aldrich M3641

Phospate buffer saline (PBS) tablet - Sigma Aldrich P4417

Stock solutions

Acrylamide/Bis (30% T, 2.67% C)

87.6 g Acrylamide (29.2 g/100 ml)

2.4 g NN – Bis – methylene-acrylamide (0.8 g /100 ml)

Made to 300 ml with distilled water. Filter and stored at 4 °C in the dark (30 days maximum).

Sample buffer (SDS reducing buffer) stored at room temperature

Distilled water 4.0 ml

0.5M Tris-HCL, pH 6.8 1.0 ml

Glycerol 0.8 ml

10% (w/v) Sodium dodecyl sulphate SDS 1.6 ml

2-β mercaptoethanol 0.4 ml

0.005% (w/v) Bromophenol blue 0.2 ml

Electrode (running) buffer (X5), pH 8.3. Stored at 4 °C

Tris base 9 g

Glycine 43.2 g

Sodium dodecyl sulphate (SDS) 3 g

Made up to 600 ml with distilled water. Warm to 37 °C before use if precipitation occurs. Dilute coml. 5X stock with 240 ml for one electrophoresis run.

Separating gel preparation

Distilled water 3.35 ml

1.5 M Tris-HCL, pH 8.8 2.5 ml

10% (w/v) SDS stock 100 ml

Acrylamide/Bis (30% stock) 4 ml

10% ammonium persulphate 50 µl

Tetramethylethylenediamine (TEMED) 5 µl

Stacking gel preparation 4% gel, 0.125M Tris, pH 6.8

Distilled water 6.1 ml

0.5 Tris-HCL, pH 6.8 2.5 ml

10% (w/v) SDS 100 µl

Acrylamide/Bis 1.3 ml

10% ammonium persulphate 50 µl

TEMED 10 µl

Transfer/equilibration buffer

Tris 3.03 g

Glycine 14.4 g

De-ionised water

Methanol v/v 200 ml

pH 8.3

Use at 4 °C

Appendix C

Companies

Axis-Shield Diagnostics Limited, The Technology Park, Dundee, DD2 1XA

Fermentas Life Sciences, Sheriff House, Sheriff Hutton Industrial Park, York YO60 6RZ

Fisher Scientific, Bishops Meadow Road, Loughborough, Leicestershire, LE11 5RG

Gene Service, 2 Cambridge Science Park, Milton Road, Cambridge CB4 0RE

Invitrogen Limited, 3 Fountain Drive, Inchinnan Business Park, Paisley PA4 9RF

Oxoid Limited, Wade Road, Basingstoke, Hampshire, RG24 8PW

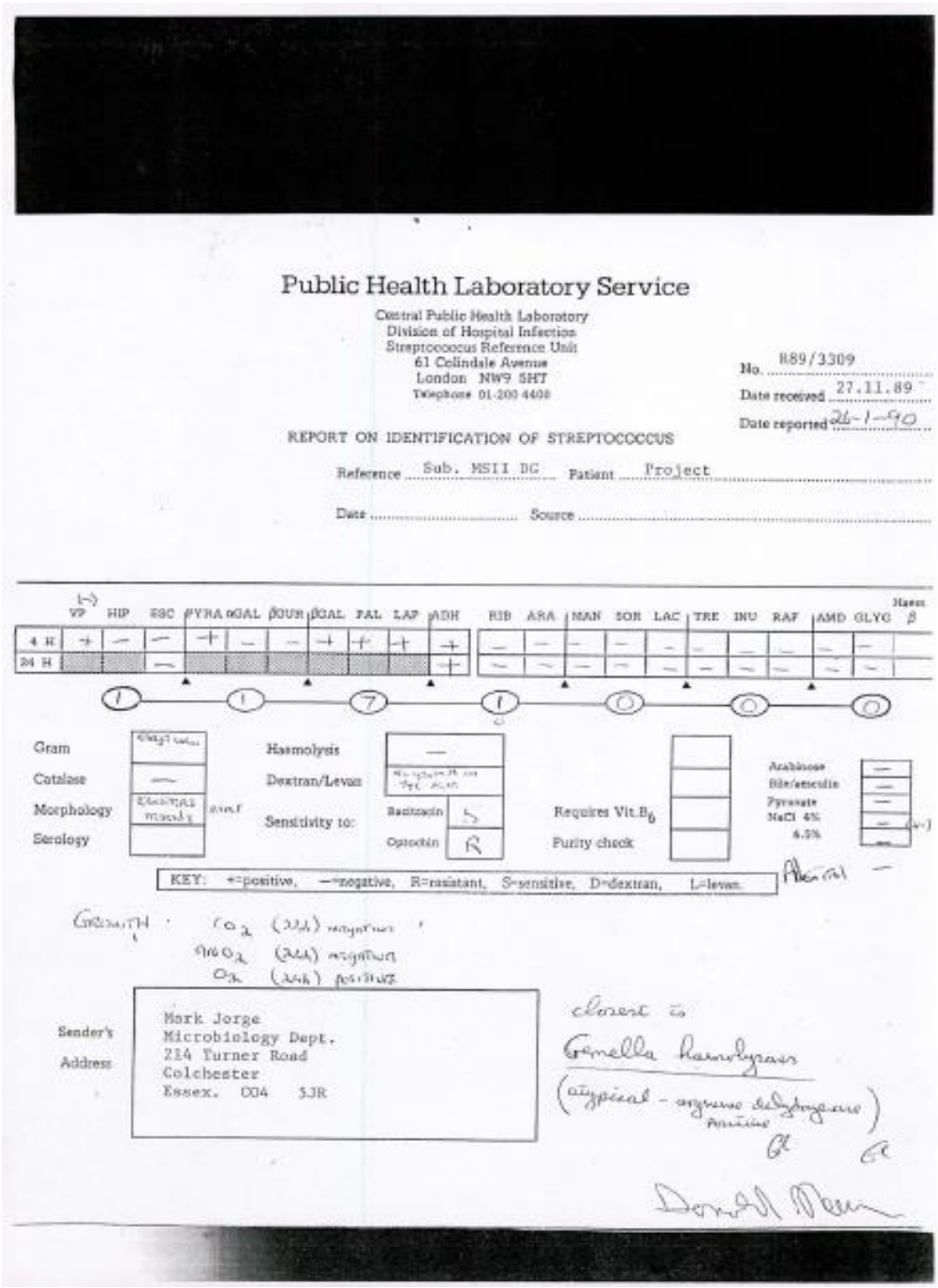
Sigma-Aldrich Company Limited, The Old Brickyard, New Road, Gillingham, Dorset, SP8 4XT

TCS Biosciences, Botolph Claydon, Buckinghamshire MK18 2LR, HBO34

Qiagen, Qiagen House, Fleming Way, Crawley, West Sussex RH10 9NQ

Appendix D

Original identification of *Dolosigranulum pigrum* 1989-1993



[redacted]

Public Health Laboratory Service

Central Public Health Laboratory
Division of Hospital Infection
Streptococcus Reference Unit
61 Colindale Avenue
London NW9 5HT
Telephone 01-200 4400

No. 889/3309
Date received 27.11.89
Date reported 26-1-90

REPORT ON IDENTIFICATION OF STREPTOCOCCUS

Reference Sub. MSII DG Patient Project

Date ..... Source .....

	VP	HIP	ESC	PYRA	αGAL	βGUR	βGAL	PAL	LAP	ADH	RIB	ARA	MAN	SOR	LAC	TRE	INU	RAF	AMD	GLYC	Haem β
4 H	+	−	−	+	−	−	+	+	+	+	−	−	−	−	−	−	−	−	−	−	
24 H			−							+	−	−	−	−	−	−	−	−	−	−	

1 1 7 1 0 0 0

Gram
Catalase
Morphology
Serology

Haemolysis
Dextran/Levan
Sensitivity to:
Bacitracin S
Optochin R

Requires Vit.B_6
Purity check

Arabinose
Bile/aesculin
Pyruvate
NaCl 4%
6.5%

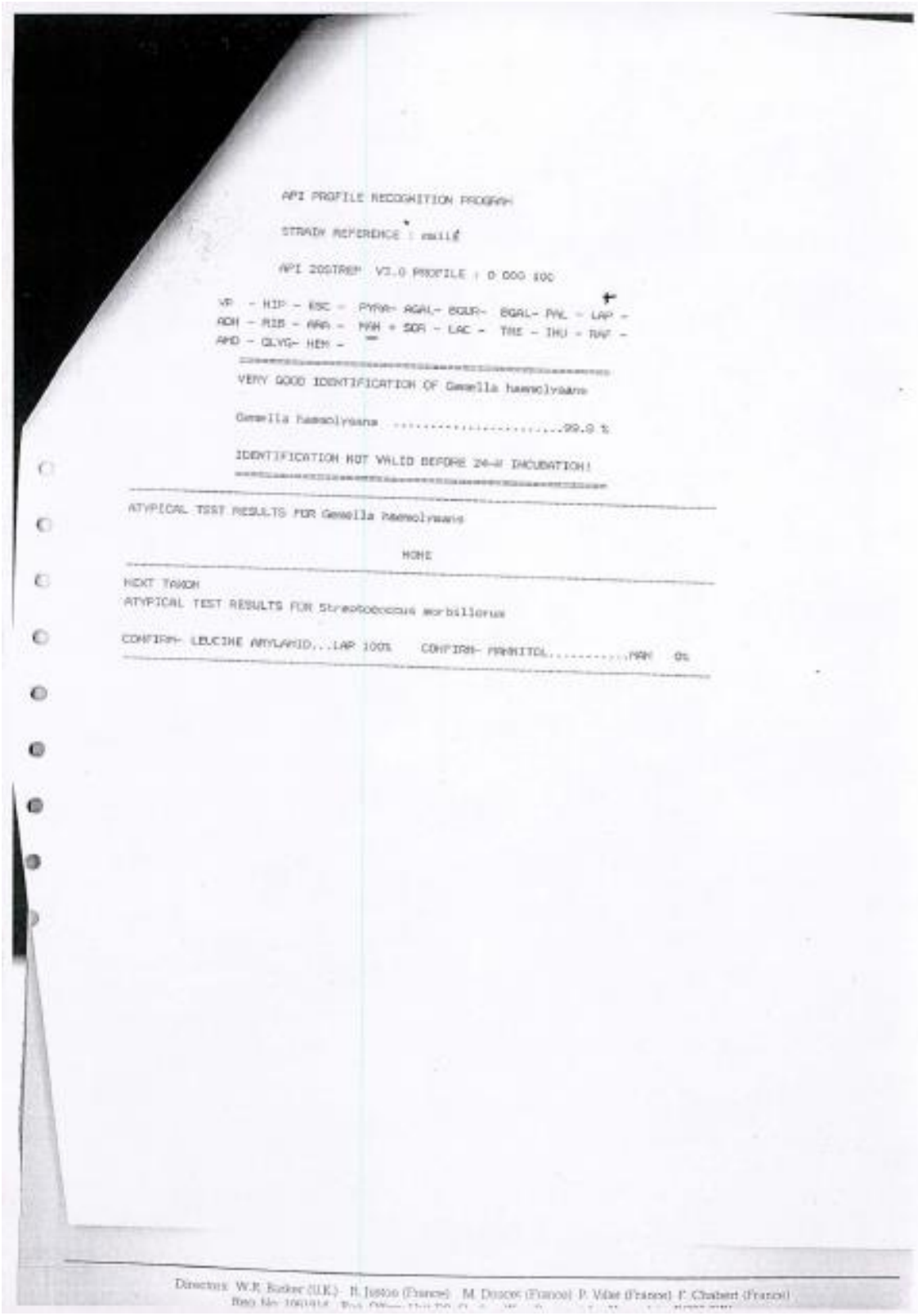
KEY: +=positive, −=negative, R=resistant, S=sensitive, D=dextran, L=levan.

GROWTH : CO_2 (22h) negative
NO_2 (24h) negative
O_2 (48h) positive

Sender's Address

Mark Jorge
Microbiology Dept.
214 Turner Road
Colchester
Essex. CO4 5JR

closest to
Gemella haemolysans
(atypical - arginine dehydrogenase positive)



API PROFILE RECOGNITION PROGRAM

STRAIN REFERENCE : coll1

API 20STREP V1.0 PROFILE : 0 000 100

VP - HIP - ESC - PYRA- AGAL- BGUR- BGAL- PAL - LAP -
ADH - RIB - ARA - MAN - SOR - LAC - TRE - INU - RAF -
AMD - GLYG- HEM -

VERY GOOD IDENTIFICATION OF Gemella haemolysans

Gemella haemolysans .....99.9 %

IDENTIFICATION NOT VALID BEFORE 24-H INCUBATION!

ATYPICAL TEST RESULTS FOR Gemella haemolysans

NONE

NEXT TAXON
ATYPICAL TEST RESULTS FOR Streptococcus morbillorum

CONFIRM- LEUCINE ARYLAMID...LAP 100% CONFIRM- MANNITOL.....MAN 0%

Directors: W.R. Barker (U.K.) H. Janton (France) M. Doucet (France) P. Vilar (France) F. Chabert (France)

Dolosigranulum pigrum	
Accession Number:	702975
Species Name:	Dolosigranulum pigrum
Strain Name:	R91/1468
Other Collection:	ATCC51524 CIP104051 IFO15550 NCFB2975
Date of Accession:	01/01/1992 00:00:00
Depositor Company:	PHLS
Type Strain:	Yes
Reference:	44:370
Isolated from:	human spinal cord, Colchester, UK
K12:	Yes
ACDP:	1
Other Catalogue Information	
Former NCFB organism	
Growth	
Growth Medium:	Todd Hewitt Borth (Oxoid)
Growth Medium No:	361
Incubation Temp:	37C
Gas Regime:	facultative anaerobic
References	
J. Appl. Bact. (1993) 75, 608-612 IJSEM (2002) 52:1122 IJSEM (2005) 55:1326 IJSEM (2005) 55:348	

http://www.ncimb.com/results.php?parent=culture

Appendix E

Preparation of anti-*Dolosigranulum pigrum* antisera

The rabbit anti-sera R3-R6 were prepared by Professor G.W.A. Dick at the Animal Unit facility of the University of Surrey under Home Office regulations. R3 and R4 were inoculated using a bacterial suspension in phosphate buffered saline (PBS) from an 8 day confluent growth of *D. pigrum* on nutrient agar (Oxoid CM3). R4 and R5 were inoculated with a suspension of cells, deposited by centrifugation, from a 6 day brain-heart infusion broth (Oxoid CMO225) culture which had been washed three times in PBS.

Immunization schedules, after a pre-immunization bleed, started with an intramuscular injection of 0.5 ml cell suspension in 0.5 ml complete Freund's adjuvant. Intravenous booster immunizations were carried out at 4 weeks and at 3 months. Sera were tested and titrated using an indirect immuno-fluorescence assay, followed by SDS-PAGE and Western Blotting. Antibody was detected, titrated and analyzed in all four rabbit sera. No reactivity was detected in pre-immune sera. Sera were absorbed with the bacterial cells, in four cycles, resulting in a progressive fall in titre. The sera were used for 2 published papers (Aguirre et al., 1993, pp. 608-612; Gay, 2013, pp. 213-232) as well as this project.

Appendix F

FORM UPR16

Research Ethics Review Checklist

University of Portsmouth

Please complete and return the form to Research Section, Quality Management Division, Academic Registry, University House, with your thesis, prior to examination

Postgraduate Research Student (PGRS) Information			Student ID:	348752
Student Name:	Mark Francis Jorge			
Department:	PHBM	First Supervisor:	Graham Mills	
Start Date: (or progression date for Prof Doc students)	2010			

Study Mode and Route:	Part-time ☐x	MPhil ☐	Integrated Doctorate (NewRoute) ☐
	Full-time ☐	MD ☐	Prof Doc (PD) ☐x
		PhD ☐	

Title of Thesis:	The potential pathogenicity of *Dolosigranulum pigrum* in multiple sclerosis, and the occurrence of the organism in the upper respiratory tract
Thesis Word Count: (excluding ancillary data)	36309

If you are unsure about any of the following, please contact the local representative on your Faculty Ethics Committee for advice. Please note that it is your responsibility to follow the University's Ethics Policy and any relevant University, academic or professional guidelines in the conduct of your study

Although the Ethics Committee may have given your study a favourable opinion, the final responsibility for the ethical conduct of this work lies with the researcher(s).

UKRIO Finished Research Checklist:
(If you would like to know more about the checklist, please see your Faculty or Departmental Ethics Committee rep or see the online version of the full checklist at: http://www.ukrio.org/what-we-do/code-of-practice-for-research/)

a) Have all of your research and findings been reported accurately, honestly and within a reasonable time frame?	YES
b) Have all contributions to knowledge been acknowledged?	YES
c) Have you complied with all agreements relating to intellectual property, publication and authorship?	YES
d) Has your research data been retained in a secure and accessible form and will it remain so for the required duration?	YES
e) Does your research comply with all legal, ethical, and contractual requirements?	YES

*Delete as appropriate

UPR 16 (2011) – August 2011

Student Statement:	
I have considered the ethical dimensions of the above named research project, and have successfully obtained the necessary ethical approval(s)	
Ethical review number(s) from Faculty Ethics Committee (or from NRES/SCREC):	08/H0303/4
Signed: *(Student)*	**Date:** 22/5/14
If you have *not* submitted your work for ethical review, and/or you have answered 'No' to one or more of questions a) to e), please explain why this is so:	
Signed: *(Student)*	**Date:**